

***Rickettsiales* and rickettsial diseases in Australia**

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary institution.

.....

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Abstract

Currently, there are 12 known *Rickettsiales* species in Australia. However research into the diversity and range of these agents in Australia is still far from complete.

A sero-epidemiological study was undertaken around the city of Launceston in Tasmania, Australia to determine the level of exposure to spotted fever group (SFG) rickettsia among the local cat and dog population. The study showed that over 50% of the dogs and cats tested were positive for SFG rickettsiae antibodies. However, no correlation was observed between the animals' health and seropositivity at the time of testing.

Ixodes tasmani ticks collected from Tasmanian devils in Tasmania were tested for the presence of SFG and typhus group (TG) rickettsiae using a specific real time PCR (qPCR), and 55% were found to be positive. The *gltA*, *rompA*, *rompB* and *sca4* genes were then sequenced. Using the current criteria this new rickettsia qualified as a *Candidatus* species, and was named *Candidatus Rickettsia tasmanensis*, after the location from which it was first detected.

Soft ticks of the species *Argas dewae* were collected from bat roosting boxes north of Melbourne. Of the ten ticks collected, seven (70%) were positive for SFG rickettsiae using the qPCR mentioned above. An isolate was obtained using cell culture isolation methods and the *rrs*, *gltA*, *rompA*, *rompB* and *sca4* genes were sequenced. Using the current criteria this new rickettsia qualified as

a novel species, and was tentatively named *Rickettsia argasii* sp. nov. after the tick genus from which it was isolated.

Four family members and their neighbour living in metropolitan Victoria became ill after exposure to a flea-infested kitten. Initial serological analysis indicated a typhus group (TG) rickettsial infection. However, testing of fleas from the group of cats in Lara, Victoria, where the kitten originated, revealed the presence of *R. felis*, the agent of cat flea typhus. This was the first case of human infection with *R. felis* in Australia and the first detection of *R. felis* in fleas in Victoria.

A tourist returning to Australia from the United Arab Emirates was diagnosed with a scrub typhus group (STG) rickettsial infection and the agent was isolated from their blood. Analysis of the *rrs* and *47kDa* genes showed significant divergence compared to all available strains of *Orientia tsutsugamushi*. Due to the degree of genetic divergence and the geographically unique origin of this isolate it was considered to be a new species, which has been tentatively named *Orientia chuto*, with 'chuto' being Japanese for 'Middle East'.

Dogs in central and northern Australia were tested for *Anaplasma platys* using a specifically designed real-time PCR (qPCR) assay. Of the 68 dogs tested, 27 (40%) were positive for *A. platys* DNA, including six dogs from Western Australia. This was the first report of *A. platys* in Western Australia.

These studies offer an insight into the range and diversity of Rickettsiales and rickettsial diseases previously unrecognised in Australia.

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The work in this thesis is my own research. Collaborating authors mentioned in chapters 3 were mainly responsible for sample collection and manuscript revision prior to submission, while in chapter 4, collaborating authors were primarily responsible for manuscript revision prior to submission. The contributions of all other parties are mentioned in the acknowledgments section of this thesis.

Original manuscripts

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Islam A, Izzard L, Unsworth N, Givney R, Ferguson J, Stenos J, Graves S. A note on rickettsiae in Australia – **Poster presentation at the 23rd Meeting of the American Society for Rickettsiology, South Carolina, USA (2009)**

Jaing J, Blacksell S, Paris D, Aukkanit N, Newton P, Phetsouvanh R, Izzard L, Stenos J, Graves S, Day N, Richards A. Diversity of the 47kDa/HtrA translated amino acid sequences among human isolates of *Orientia tsutsugamushi* – **Poster presentation at the 23rd Meeting of the American Society for Rickettsiology, South Carolina, USA (2009)**

Abbreviations

AG – Ancestral group

AGRF – The Australian Genomic Research Facility

ASF – Australian spotted fever

ATF – Australia tick fever

BHQ-1 – Black hole quencher-1

Bp – Base pairs

CCFM – Cell culture freezing media

CF – Complement Fixation

C_t – Threshold cycle

DNA – Deoxyribonucleic acid

dNTP – Deoxyribonucleic triphosphate

EDTA – Ethylenediaminetetraacetic Acid

ELISA – Enzyme-linked immuno sorbent assay

FISF – Flinders island spotted fever

FITC – Fluorescein isothiocyanate

gltA – Citrate synthase gene

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IFA – Immunofluorescent assay

kDa – Kilodalton

NCBI – National Centre for Biotechnology Information

NSW – New South Wales

NTC – No template control

OPNP – Organ Pipes National Park

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

QLD – Queensland

qPCR – Real-time PCR

QTT – Queensland tick typhus

RBC – Red blood cell

RNA – Ribonucleic acid

rompA – Rickettsial outer membrane protein A gene

rompB – Rickettsial outer membrane protein B gene

RPM – Revolutions per minute

rRNA – Ribosomal RNA.

rrs – 16S ribosomal RNA gene

sca4 – The rickettsial gene D / PS–120 gene

SDS – Sodium dodecyl sulphate

SFG – Spotted fever group

TAE – Tris-acetate-EDTA

TG – Typhus group

T_m – Melting temperature

TRG – Transitional group

Chapter 1. Literature Review

1.1. Introduction

In 1909 Howard T Ricketts first described small organisms that appeared to be associated with the disease Rocky Mountain spotted fever²⁰⁹. This was the first report of the organism *Rickettsia*. The diseases associated with these organisms however have been described in literature dating back possibly as far as 1083, with typhus extensively described by Fracastoro in 1546²⁰⁴.

In 1910 Theiler proposed the new genus and species *Anaplasma marginale* as the aetiological agent of anaplasmosis, found in the erythrocytes of cattle²⁴⁹ and in 1916 Rocha Lima proposed the genus name *Rickettsia*. At this stage the only species in the genus was the agent of epidemic typhus *Rickettsia prowazekii*¹⁸⁷.

By 1953, the family *Rickettsiaceae* contained 4 genera, namely *Rickettsia*, *Ehrlichia*, *Cowdria*, and *Coxiella*. The genus *Rickettsia* contained seven species: *R. prowazekii*, *R. typhi*, *R. tsutsugamushi*, *R. rickettsii*, *R. conorii*, *R. australis* and *R. akari*. The genus *Ehrlichia* contained *E. bovis*, *E. canis*, *E. ovina* and *E. kurlovi*. *Cowdria* and *Coxiella* each only contained one species, *Cowdria ruminantium* and *Coxiella burnetii* respectively¹⁸⁷.

In 1974 the eighth edition of Bergey's manual of determinative bacteriology was published. By this stage the order *Rickettsiales* contained three families; *Rickettsiaceae*, *Bartonellaceae* and *Anaplasmataceae*. The family *Rickettsiaceae* contained among others the genus *Rickettsia* (which included the species *R. tsutsugamushi*), *Ehrlichia* and *Coxiella*, while the genus *Anaplasma* was within the *Anaplasmataceae* family¹⁵⁸.

In 1989, *Coxiella burnetii* was removed from the *Rickettsiaceae* family after 16S gene sequencing revealed that it was more closely related to the genus *Legionella* than *Rickettsia*²⁷⁷. This was followed by the removal of *Bartonella* spp. in 1993²⁹.

The species *R. tsutsugamushi* was moved into the novel genus *Orientia* in 1995 and renamed *Orientia tsutsugamushi*²⁴⁴.

In 2001 the genera within the *Rickettsiaceae* and *Anaplasmataceae* underwent extensive reorganisation with the help of molecular analysis. The tribes *Rickettsiae*, *Ehrlichieae* and *Wolbachieae* were removed. The genus *Wolbachia* was transferred from the *Rickettsiaceae* to the *Anaplasmataceae* family. Several species of *Ehrlichia* and *Anaplasma* were regrouped into various genera⁶⁶.

The order *Rickettsiales* currently contains six distinct families including the families *Anaplasmataceae* and *Rickettsiaceae* (Figure 1). The family *Anaplasmataceae* is broken into five genera; *Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* while the family *Rickettsiaceae* is separated into two genera; *Rickettsia* and *Orientia*. *Rickettsia* was traditionally broken up into spotted fever group (SFG), typhus group (TG) and ancestral group (AG), although recent articles have suggested the formation of a 4th group called the transitional group (TRG) (Figure 1)⁹¹.

Although there are several genera within the order *Rickettsiales* this thesis focused on *Rickettsia*, *Orientia* and *Anaplasma* as these genera are the cause of most rickettsial infections of humans and animals in Australia.

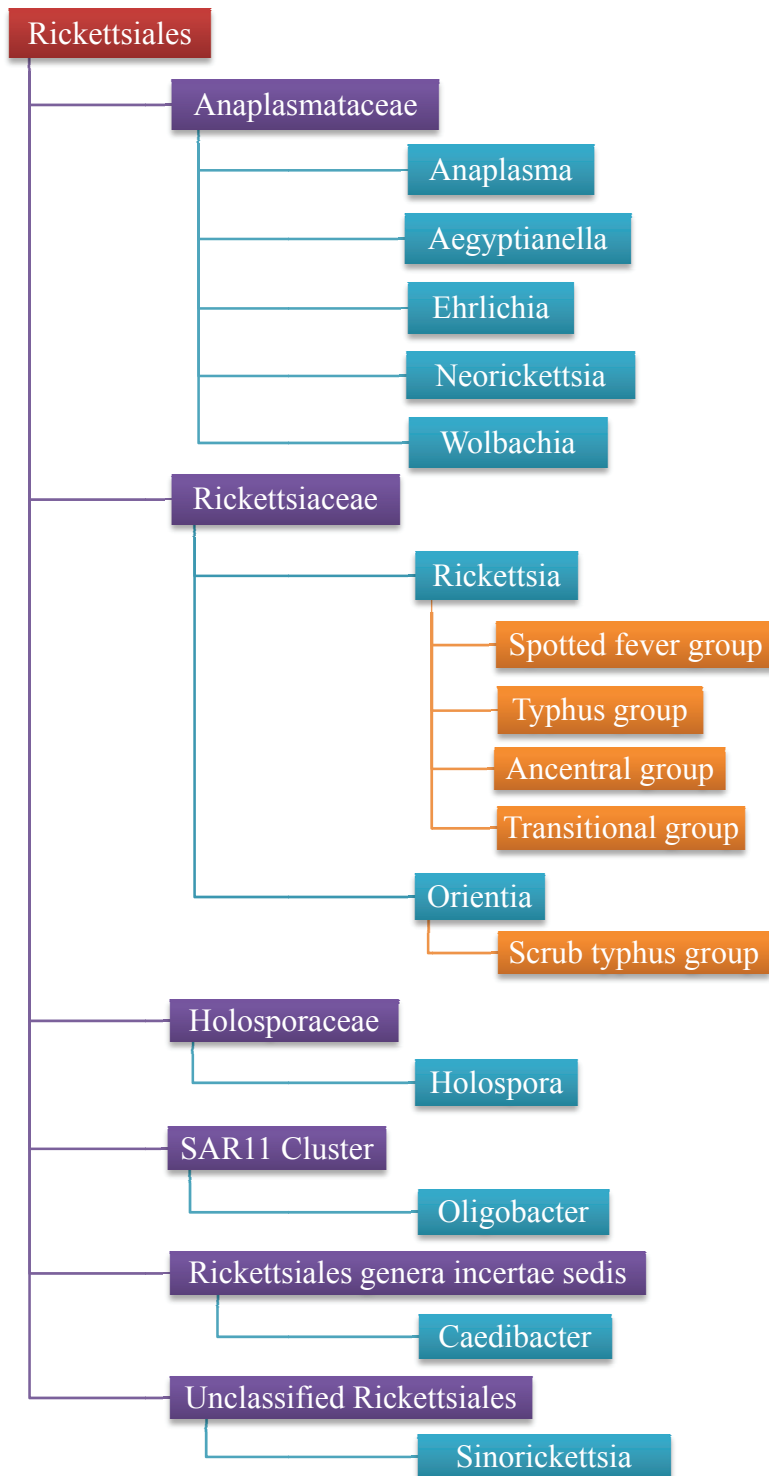


Figure 1. Phylogenetic relationship of members of the order *Rickettsiales* adapted from the Taxonomy browser within the NCBI website (<http://www.ncbi.nlm.nih.gov>) showing the order (red), family (purple), genus (blue) and species group (orange).

1.2. Taxonomy and Pathogenicity of the order

Rickettsiales

1.2.1. *Anaplasmataceae*

1.2.1.1. *Anaplasma*

Anaplasma are small Gram-negative-like coccoidal cells 0.2 – 0.4 µm in diameter. They survive within vacuoles in the cytoplasm of eukaryotic cells and divide by binary fission. Each vacuole can contain numerous organisms²¹¹.

Currently there are six validated species of *Anaplasma* (Table 2), all of which infect animal and/or human haematopoietic cells^{31, 211}. The three species; *A. marginale*, *A. centrale* and *A. ovis*, infect erythrocytes^{31, 134, 211}; *A. phagocytophilum* primarily infects neutrophils^{31, 158, 211}; *A. bovis* infects monocytes³¹; and *A. platys* infects megakaryocytes and platelets^{31, 232}.

Symptoms caused by *Anaplasma* spp. vary from species to species, and include anaemia, leukopaenia, thrombocytopenia and anorexia^{31, 172, 211}.

Anaplasma spp. are not transovarially transmitted in ticks, meaning they act as vectors of *Anaplasma* but not reservoirs, with vertebrate animals being the primary reservoirs³¹. For example white tailed deer are considered to be a natural reservoir for *Anaplasma phagocytophilum*^{65, 246}.

Ticks involved with transmission of *Anaplasma* vary from species to species and from country to country with *Ixodes*, *Rhipicephalus*, *Dermacentor*³¹ and *Amblyomma*⁷⁴ ticks being some of the species recognised as vectors.

1.2.1.2. *Ehrlichia*

The size and shape of *Ehrlichia* species are similar to *Anaplasma*, and like *Anaplasma*, the target cells for *Ehrlichia* are primarily haematopoietic cells. *E. chaffeensis*, *E. canis* and *E. muris* infect monocytes^{31, 211} and *E. ewingii* infects neutrophils²³². The exception is *E. ruminantium* (previously known as *Cowdria ruminantium*) which infects both neutrophils and endothelial cells^{31, 211}. They survive within membrane-surrounded vacuoles derived from an initial endosome in the cytoplasm of eukaryotic cells. The vacuoles differ to *Anaplasma* however, in that they are filled with fibrillar material¹⁹⁴.

As with *Anaplasma*, illness due to *Ehrlichia* is usually a result of damage to the cells that the organisms are infecting, resulting in leukopaenia, thrombocytopenia and, depending on the species, anaemia¹⁷⁹. Other generalised symptoms include fever, headache, myalgia, and arthralgia¹⁷⁰.

To date, the only known vectors for *Ehrlichia* are ticks, and like *Anaplasma* they cannot be transovarially transmitted and therefore a number of mammals are the presumed reservoirs for *Ehrlichia* including white tailed deer, domestic dogs, wolves and goats^{31, 87, 179}.

The species of ticks involved with transmission vary, although as most ehrlichial infections of animals are asymptomatic, there is limited clinical data available^{31, 211}. Some of the known tick genera that harbour this agent include

Amblyomma^{13, 31, 232}, *Rhipicephalus*^{161, 232}, *Dermacentor*²³², *Haemaphysalis*¹²⁶ and *Ixodes*⁷².

Some *Ehrlichia* species however appear to be quite specific for their tick vectors, for example *E. canis* appears to be primarily transmitted by *Rhipicephalus sanguineus* ticks^{161, 232}, while the only known vectors of *E. ruminantium* are *Amblyomma* sp. ticks³¹.

1.2.2. ***Rickettsiaceae***

1.2.2.1. *Rickettsia*

Rickettsiae are Gram-negative-like coccobacilli approximately 0.8 to 2µm in length and 0.3 to 0.5µm in diameter¹⁷². Like *Anaplasma* and *Ehrlichia*, *Rickettsia* are obligate intracellular organisms that survive in the cytoplasm, and in the case of SFG rickettsiae, the cytoplasm and nucleus of eukaryotic cells¹⁹⁰. However, unlike *Anaplasma* and *Ehrlichia*, *Rickettsia* do not form vacuoles¹⁵⁸.

To date, there are 18 SFG, 2 TG 2 Ancestral group (AG), and 3 Transitional group (TRG) species of *Rickettsia* that have been validated (Table 1), many of which cause disease in humans and animals¹⁷⁹. *Rickettsiae* are able to infect any nucleated cell in the host, therefore their primary targets are the cells with which they first come into contact²⁶⁸. As the organism spreads through the blood, endothelial cells tend to be the primary target, especially within vessels in the brain and lungs²⁶². Illness from rickettsial infection is a result of damage to the endothelial cells caused by an increased cell membrane permeability and/or attack by the host's immune system and/or apoptosis. This in turn increases the permeability of the blood vessels resulting in leakage of intravascular fluid into

the surrounding extra-vascular space²⁶⁹. Although rickettsial infection can affect every organ, the organism does not commonly spread beyond the vascular and lymphatic systems²⁶⁸.

As with *Anaplasma* and *Ehrlichia*, *Rickettsia* are transmitted by an arthropod vector, primarily ticks, although unlike *Anaplasma* and *Ehrlichia*, ticks typically act as both vector and reservoir for rickettsiae. Tick species from the genus *Amblyomma*¹³⁷, *Bothriocroton* (formally *Aponomma*)²³⁷, *Haemaphysalis*¹³⁷, *Dermacentor*^{157, 164}, *Rhipicephalus*²⁵⁴, *Ixodes*⁴¹ and *Argas*¹⁸⁸ have all been associated with rickettsial species. Chigger mites (*Leptotrombidium deliense*)²⁵⁴, fleas (*Ctenocephalides* spp.)^{195, 221} and lice (primarily *Pediculus humanus*)¹⁵⁸ have been recorded as vectors of various rickettsial species.

1.2.2.2. *Orientia*

Like all *Rickettsiales*, *Orientia* are obligate intracellular organisms. They are rod shaped cells approximately 1.2 to 3.0µm in length and 0.5µm in width²⁷¹ and until recently were considered to be a species of *Rickettsia*²⁴⁴.

The genus currently contains only the single species *Orientia tsutsugamushi*, although the species is broken into several serotypes including the three original serotypes; Kato, Karp and Gilliam²⁷¹.

As with *Rickettsia*, the primary site of infection is the vascular endothelial cells¹⁵⁹, but, *Orientia* can also infect leukocytes²¹⁰ including monocytes and macrophages⁴³. As with rickettsial disease, *Orientia* infection causes an increase in vascular permeability due to damage to the vascular endothelial cells, resulting in an increase in extra-vascular fluid and subsequently

inflammation around the brain and fluid in the lungs, and, in severe cases, multi-organ failure²⁶⁶.

The vector of *Orientia tsutsugamushi* is the larval trombiculid mite (*Leptotrombidium* spp.). The vector species vary from region to region, however the primary species are *L. deliense*, *L. fletcheri* and *L. arenicola*²²⁵.

1.3. Interactions of *Rickettsiales* and arthropods

The route of transmission of all species of *Anaplasma*, *Ehrlichia* and *Rickettsia* is via an arthropod vector²⁴⁸. The hard ticks (Ixodidae) are the primary vectors for *Anaplasma*, *Ehrlichia* and the spotted fever and transitional group rickettsia, with the exception of *R. akari* and *R. felis*, which are transmitted by mites and fleas respectively^{2, 113, 248}. Typhus group rickettsiae are transmitted by fleas while scrub typhus group rickettsiae are transmitted by trombiculid mites^{220, 225}. Infection in vertebrates is typically via a bite from an infected arthropod²⁴⁸.

Only selected animals produce rickettsemias of sufficient extent and duration to allow uninfected ticks to become infected, for example, *Anaplasma phagocytophilum* in the white tailed deer^{65, 246}. For the majority of rickettsial species, the tick vector is also the reservoir for the organism and these are maintained by inheritance of the agent, specifically the tick's progeny are born infected due to transovarial transmission²⁴⁸. Uninfected ticks however are believed to primarily acquire rickettsiales by co-feeding, when a number of ticks feed within close proximity resulting in direct spread²⁴⁸.

1.4. Genomics of the order *Rickettsiales*

Genetically, the species *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Orientia* have one major trait in common, and that is the small size of their genomes (between 1.1 to 1.5Mb)¹⁶⁷ with G+C content of approximately 30%¹⁴. Even with such a small genome, as much as 24% of the DNA of some *Rickettsiales* species is non-coding DNA or pseudogenes⁵. This is believed to be a result of genome degradation and reduction accompanying adaptation to a parasitic intracellular lifestyle²². Many biosynthetic pathways present in free-living bacteria are

replaced by transport systems in *Rickettsiales*, resulting in a complete dependence upon the host cell for survival²⁰⁷.

Horizontal gene transfer is a common practice between prokaryotic organisms. As *Rickettsiales* are obligate intracellular organisms, they rarely come into contact with microorganisms of other species and therefore have little opportunity to exchange DNA¹⁶⁷.

In 2005 Ogata reported on the presence of two plasmids within the species *R. felis*. This was the first report of a plasmid within a *Rickettsiales*¹⁶⁸. Plasmids have since been detected in *R. helvetica*, *R. peacockii* and *R. massiliae* along with a number of non-validated species¹⁴.

Members of the genus *Rickettsia* are phylogenetically the closest microbial relative to the eukaryotic mitochondria, with speculation that they evolved from a common ancestor⁶.

Table 1. Current list of validated rickettsial species.

Species	Group	Common Vector	Diseases	Reference
<i>R. aeschlimannii</i>	SFG	<i>Hyalomma marginatum</i>	Unnamed Disease	16
<i>R. africae</i>	SFG	<i>Amblyomma</i> spp.	African tick bite fever	131
<i>R. asiatica</i>	SFG	<i>Ixodes ovatus</i>	Unknown	85
<i>R. conorii</i>	SFG	<i>Rhipicephalus sanguineus</i>	Mediterranean spotted fever	36
<i>R. heilongjiangensis</i>	SFG	<i>Dermacentor silvarum</i>	Unnamed Disease	284
<i>R. helvetica</i>	SFG	<i>I. ricinus</i>	Unnamed Disease	17
<i>R. honei</i>	SFG	<i>Bothriocroton hydrosauri</i>	Flinders Island spotted fever	240
<i>R. japonica</i>	SFG	<i>Dermacentor</i> , Ixodes and <i>Haemaphysalis</i> spp.	Oriental spotted fever	256
<i>R. massiliae</i>	SFG	<i>Rhipicephalus</i> spp.	Unnamed Disease	18
<i>R. montanensis</i>	SFG	<i>Dermacentor</i> spp.	Unknown	19
<i>R. parkeri</i>	SFG	<i>Amblyomma</i> spp.	Maculatum disease	138, 268
<i>R. peacockii</i>	SFG	<i>D. andersoni</i>	Unknown	164
<i>R. raoultii</i>	SFG	<i>Dermacentor</i> spp.	Unknown	157
<i>R. rhipicephali</i>	SFG	<i>R. sanguineus</i>	Unknown	38
<i>R. rickettsii</i>	SFG	<i>D. andersoni</i>	Rocky Mountain spotted fever	281
<i>R. sibirica</i>	SFG	<i>D. silvarum</i>	Siberian tick typhus	283
<i>R. slovaca</i>	SFG	<i>D. marginatus</i>	Tick-borne lymphadenopathy	224
<i>R. tamurae</i>	SFG	<i>A. testudinarium</i>	Unknown	83
<i>R. prowazekii</i>	TG	<i>Pediculus humanus humanus</i>	Epidemic Typhus	55
<i>R. typhi</i>	TG	<i>Xenopsylla cheopis</i>	Murine/Endemic Typhus	187
<i>R. bellii</i>	AG	<i>Dermacentor</i> spp.	Unknown	188
<i>R. canadensis</i>	AG	<i>H. leporispalustris</i>	Unknown	156
<i>R. akari</i>	TRG	<i>Allodermanyssus sanguineus</i>	Rickettsialpox	113
<i>R. australis</i>	TRG	<i>Ixodes</i> spp.	Queensland tick typhus	9
<i>R. felis</i>	TRG	<i>Ctenocephalides felis</i>	Cat flea rickettsiosis	107

Table 2. Current list of validated *Anaplasma* and *Ehrlichia* species.

Species	Common Vector	Common Pathogenic Host	Disease	Reference
<i>A. bovis</i>	<i>Haemaphysalis</i> sp., <i>Rhipicephalus</i> sp. and <i>Amblyomma</i> sp.	Bovine	Tick Fever	62
<i>A. central</i>	<i>Ixodes</i> sp. and <i>Haemaphysalis</i> sp.	Bovine	Tick Fever	250
<i>A. marginale</i>	<i>Ixodes</i> sp., <i>Dermacentor</i> sp. and <i>Boophilus</i> sp.	Ruminant	Tick Fever	249
<i>A. ovis</i>	<i>Dermacentor</i> sp.	Ruminant	Unnamed Disease	143
<i>A. platys</i>	<i>R. sanguineus</i>	Canine	Canine infectious cyclic thrombocytopenia	102
<i>A. phagocytophilum</i>	<i>Ixodes</i> sp. and <i>Dermacentor</i> sp.	Canine, Ruminant, Human, Others	Human granulocytic anaplasmosis Tick-borne fever	79
<i>E. canis</i>	<i>R. sanguineus</i>	Canine	Canine monocytic ehrlichiosis	61
<i>E. chaffeensis</i>	<i>Amblyomma americanum</i> and <i>Dermacentor</i> sp.	Canine, Human	Human monocytic ehrlichiosis	3
<i>E. ewingii</i>	<i>A. americanum</i>	Canine, Human	Canine granulocytic ehrlichiosis <i>E. ewingii</i> granulocytic ehrlichiosis	4
<i>E. muris</i>	<i>H. flava</i>	Mice, Voles	Unknown	278
<i>E. ovina</i>	Unknown	Ovine	Unnamed Disease	144
<i>E. ruminantium</i>	<i>Amblyomma</i> spp.	Ruminant	Heartwater fever	47

1.5. Methods of identification and characterisation of *Rickettsiales*

1.5.1. Staining

Organisms in the order *Rickettsiales* are considered Gram-negative-like, however they stain very poorly with the Gram staining method¹³⁶ and are typically stained using Giemsa or Gimenez staining methods⁹². This technique works particularly well with members of the *Anaplasmataceae* family, as they form clusters within vacuoles in the host cell and are therefore quite clearly visible when stained^{31, 76}. The exception to this is *Anaplasma platys*, which is often difficult to detect due to low or absent numbers during certain stages of its cyclic parasitaemia^{28, 101, 148}. Immunohistochemical staining of biopsy samples can also be an effective method of rickettsial detection; however this method has limited specificity in detecting different species¹⁷⁶.

1.5.2. Serology

The first serological method for detection of rickettsial disease (the Weil-Felix test) was designed in 1916²⁷⁶. This method relied on the cross-reaction of rickettsial antibodies with *Proteus vulgaris* strains OX2 and OX19 for SFG and TG and *P. mirabilis* strain OXK for STG rickettsia²⁰¹. This test however has both low sensitivity and specificity⁹⁷. Rickettsial antibodies in human and animal specimens have since been detected using various methods including complement fixation (CF)²²⁹, enzyme-linked immunosorbent assay (ELISA)¹⁰⁰ and immunofluorescence assay (IFA)⁹⁷. Of these, IFA is considered the reference method for the detection of antibodies to both *Rickettsiaceae* and

Anaplasmataceae^{136, 179, 181}. The main limitations with this method are the degree of cross-reactivity between species of the same genus¹⁸¹, and that it is a retrospective analysis, as it can take several weeks to detect antibodies, usually well after the patient has recovered.

1.5.3. Isolation

Isolation of *Rickettsiales* from a clinical sample is the most definitive diagnostic method for detection of a rickettsial infection¹⁸¹. It can be performed using various samples including buffy coat from heparinised or EDTA-treated whole blood, triturated blood clot, skin biopsy and necropsy tissue. Alternatively, if a suspect arthropod is available, cultures can be performed directly on it^{136, 181}. In the past animal inoculation and yolk sac culture were the commonly used isolation techniques^{48, 98}. Cell culture methods are now the main techniques used for isolation of *Rickettsiaceae* and *Anaplasmataceae*^{106, 136}, although animal inoculation is still useful for the removal of contaminants, as cell cultures are typically antibiotic-free⁷¹. Cell lines used include Vero, L929, HEL, XTC-2, MRC5 and DH-82^{54, 136, 181}. Again, this method is retrospective, as it can take weeks to obtain an isolate, usually well after the patient has recovered.

1.5.4. Polymerase Chain Reaction (PCR)

Clinical manifestations of rickettsial disease are typically non-specific; therefore laboratory testing of samples is crucial for accurate diagnosis. Typically this is performed by serology, although this method has limitations, with antibodies not present at the early stages of infection and a degree of cross-reactivity among species^{77, 78}. Advancements in molecular methods have allowed the

development of highly specific, sensitive and rapid assays for detection of *Rickettsiales* in many different samples including blood, tissue and arthropods^{77, 78}. The key advantage to this assay is its speed, as it can be performed in one day.

1.5.4.1. Conventional PCR

The first PCR to detect *Rickettsia* was published in 1989 and targeted the 17kDa gene (*orf17*)²⁵⁵. Since then, assays have been developed that target a number of rickettsial genes. These include the 16S rRNA gene (*rrs*)²¹⁶, citrate synthase gene (*gltA*)²¹⁸, OmpA gene (*rompA*)⁸², OmpB gene (*rompB*)²¹⁷ and gene D (*sca4*)²²³. Assays targeting the 16S rRNA gene¹⁶⁹, 22kD, 47kD, 110kD, groESL and 56kD genes for *Orientia tsutsugamushi* have also been designed¹²⁸. Assays have also been developed that amplify various genes including the 16S rRNA, groESL, surface protein⁶⁶, citrate synthase¹¹⁵, *msp2*⁶⁴ and “*dsb*” genes⁶³ of the family *Anaplasmataceae*.

1.5.4.2. Real-time PCR (qPCR)

Real time PCR (qPCR) involves the use of a fluorescent probe to detect the amplification of target DNA as it occurs. One common method used for rickettsial identification is the TaqMan probe method¹⁰⁵. This method has been successfully used to detect members of the *Anaplasmataceae*¹⁹⁶ and *Rickettsiaceae*¹²² families. This is considered one of the most sensitive and specific assays and is the fastest way to diagnose an infection. Therefore it is often the molecular method of choice.

1.5.4.3. *Molecular speciation*

Initially, 16S gene analysis was used for speciation of *Rickettsiales*^{116, 216}. With the increasing number of gene targets, molecular methods involving comparison of multiple genes were developed²⁰⁶, but it was not until 2003 that a widely recognised molecular criteria for the speciation of *Rickettsiae* was published⁸¹. It specified minimum and maximum percentage homologies of 5 genes required to classify an isolate into a genus and species (Figure 2). No such guideline has been published to speciate members of the *Anaplasmataceae* family, although a preliminary guideline was used in 2001 to reorganise the family using 16S rRNA, groESL and surface protein genes⁶⁶.

Furthermore, all members of the *Rickettsiales* require that an isolate be deposited into a culture collection to be defined as a novel species. Until this has been achieved the new agent is referred to as a “Candidatus” species¹⁶⁰.

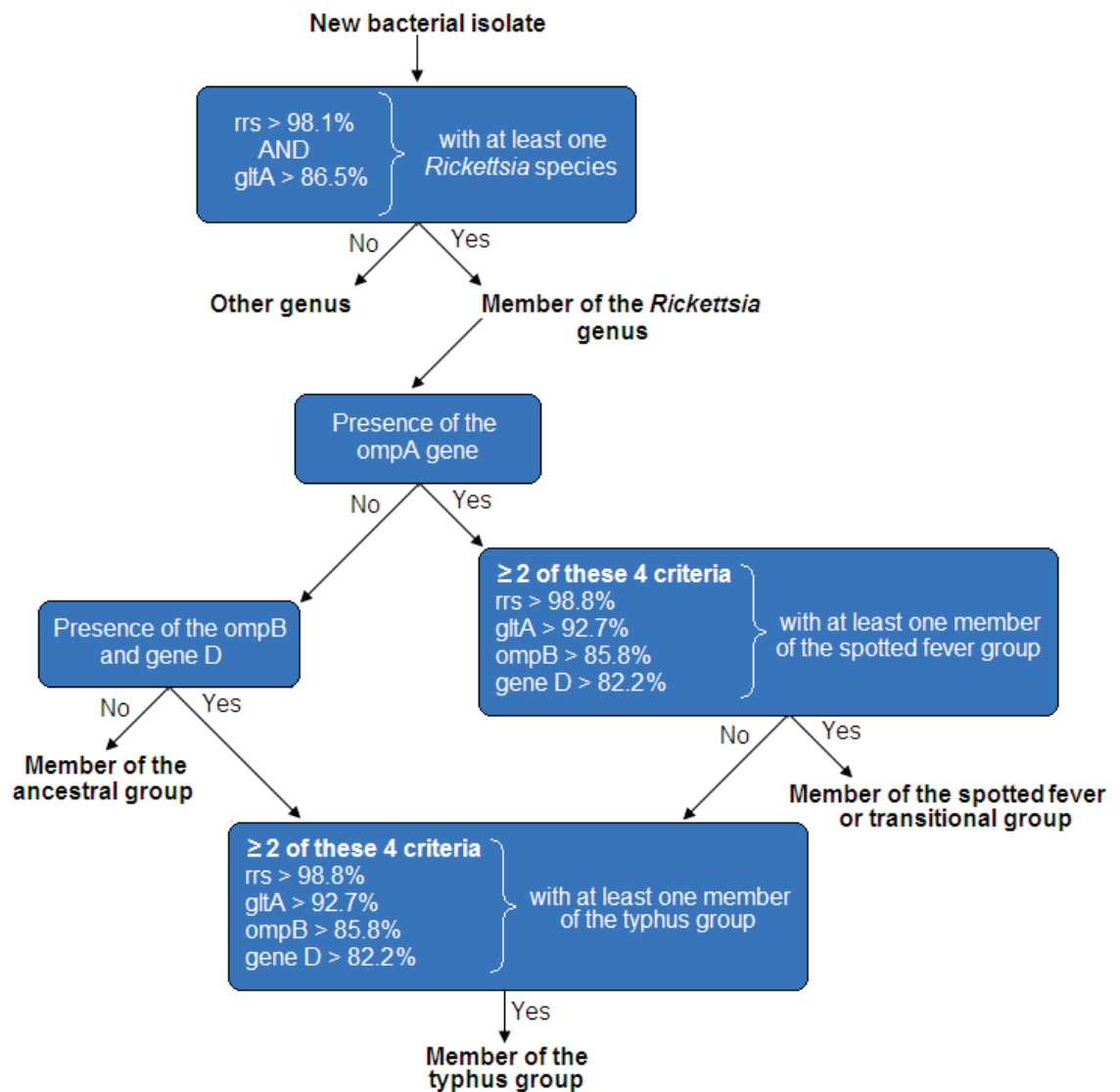


Figure 2. Flow diagram of phylogenetic classification of *Rickettsia* adapted from Fournier et al.⁸¹

1.6. Geographic distribution of the *Rickettsiales*

Members of the genus *Rickettsia* are found on every continent in the world that has been examined²⁰³. *Orientia tsutsugamushi* is endemic to a 13 million square kilometre triangle-shaped area of the Asia-Pacific rim from Afghanistan in the north west, across to Kamchatka peninsula, Russia in the north east, down to northern Australia in the south^{129, 271}. Like *Rickettsia*, species of the family *Anaplasmataceae* are also found on every continent examined^{33, 87}.

1.7. *Rickettsiales* species in Australia

1.7.1. *Anaplasma*

Three species of *Anaplasma* are known to occur in Australia. They are *Anaplasma platys*³³, *Anaplasma marginale* and *Anaplasma centrale*²¹⁵. Both *A. platys* and *A. marginale* are naturally occurring in northern Australia, while a low virulence strain of *A. centrale* was imported from South Africa in 1934 for use as a live vaccine against *A. marginale* infection in cattle²¹⁵.

1.7.1.1. Australian (cattle) tick fever (*Anaplasma marginale*)

Australian tick fever (ATF) is a disease of cattle in northern Queensland caused primarily by the agents *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*¹²⁴, with infection percentages of 81.5, 11.0 and 7.5 respectively²⁴, although other estimates put the percentage of cases caused by *A. marginale* at more than 13%²³.

Believed to have been imported into Australia with *Boophilus microplus* ticks on cattle in the 19th century²⁶, the economic cost to the country has been great. In 1998 it was estimated that the annual cost in lost farming profits due to tick fever was around \$22 million²⁶.

The effectiveness of the *A. centrale* vaccine is quite variable in Australia and worldwide²⁶. Immunity to *A. marginale* by inoculation with *A. centrale* is believed to be due to the antigenic similarities between them resulting in cross protection from the animal's immune system¹. A new vaccine using a low virulent Australian isolate of *A. marginale* has been trialled²³. The study indicated that this may serve as a useful vaccine in Australia, although more research into the safety and potency of the vaccine is required before it can be used commercially²³.

Even with the estimated financial impact of this organism and the availability of commercial vaccines, only around 33% of beef farmers in high risk areas of Australia vaccinate against tick fever²⁵.

1.7.1.2. Canine infectious cyclic thrombocytopenia (*Anaplasma platys*)

In 2001, the agent of canine infectious cyclic thrombocytopenia (*A. platys*) was first detected in Australia in free-roaming dogs from the Northern Territory³³. This was the first report of a member of the *Anaplasmataceae* infecting dogs in Australia and was reported as being “among the most exciting discoveries within the area of canine infectious diseases in Australia in the past decade”¹¹⁷.

The brown dog tick (*Rhipicephalus sanguineus*) was always believed to be the only vector for the transmission of *A. platys*, and the rate of infection among dogs altered with the seasonal effects on tick populations and life cycles³⁴. In Australia however, the rate of infection among dogs did not correlate with the seasonal levels of *R. sanguineus* ticks. This suggested the presence of a second vector³⁴. In 2005 Brown *et al.* discovered that a species of chewing lice (*Heterodoxus spiniger*) collected from dogs contained *A. platys* DNA. He described these lice as the second vector for *A. platys* in Australia³⁴.

1.7.2. *Ehrlichia*

As yet, no evidence of an established *Ehrlichia* species has been documented in Australia, although a few reports of the high potential of ehrlichial infection spread in Australian quarantine facilities have been published. These reports describe the potential risks of serologically positive horses being imported into Australia²³⁶ and the potential spread of infection among dogs and native wildlife by *Ehrlichia* infected ticks²³⁵.

Symptoms consistent with *Anaplasmataceae* infection have been noted by veterinarians in the Northern Territory for many years³³. *Ehrlichia canis* was the suspected agent and several serological surveys to investigate *Ehrlichia canis* in dogs were undertaken, with no success^{88, 149}. It was later determined that serological cross-reactions in these studies were caused by *A. platys*.

1.7.3. *Rickettsia*

The first rickettsial species identified in Australia was *R. prowazekii*, which was brought to Australia by convict and immigrant ships from as early as the first

fleet (1788) to 1868⁴⁹. The disease was never able to establish a foothold in Australia, probably due to the different climate and cleaner living conditions⁹⁵, although several outbreaks were reported in new settlements in the early days in every state except Western Australia, with the last outbreak occurring in the central Victorian goldfields in 1853⁴⁹. Cases of Brill-Zinsser disease (recrudescence of latent *R. prowazekii* disease) have occurred in Australia since then, although in each case *R. prowazekii* was originally contracted overseas^{183, 251}. More cases of Brill-Zinsser disease can be anticipated as people still migrate to Australia from regions that are endemic for *R. prowazekii*⁹³.

Three rickettsial species known to cause human disease are endemic in Australia, *Rickettsia typhi*⁸⁶, *R. australis*⁹ and *R. honei*⁹⁸. The first diagnosed case of *R. typhi* was in South Australia in 1913. Since this time it has been detected in Queensland²⁷⁹, New South Wales²⁴⁷, Western Australia²²⁰, and, more recently, Victoria¹²³. It is likely that this organism is present Australia-wide.

Historically, *R. australis* was believed to only occur in northern Queensland and consequently was named “North Queensland Tick Typhus”¹³³. It is now known to occur as far north as the Torres Strait islands²⁵⁸, and down the east coast of Australia as far south as Gippsland, Victoria⁶⁷.

The agent of Flinders Island spotted fever (*R. honei*) was believed to be present only on a small island off the coast of Tasmania in the south east of Australia. It has now been shown to be more widespread, with cases occurring in South Australia and Tasmania²⁶⁰. More recently a new strain (*R. honei* strain

marmionii) was detected in Victoria²⁵⁷, Queensland, South Australia, Tasmania²⁵⁹ and the Torres Strait Islands²⁵⁸.

Cases of infection with other rickettsial agents have been reported including *R. africae*²⁷⁰, and *R. conorii*⁹⁹, although the infections were all in patients returning from overseas

R. felis has been detected in cat fleas in Western Australia²²¹ and more recently Victoria, with suspected human cases being reported (Chapter 6).

Several SFG rickettsiae of unknown pathogenicity have been detected in Australian ticks. These include ‘*Candidatus R. gravesii*’ isolated from *Amblyomma triguttatum* ticks in Western Australia¹⁷⁵, ‘*Candidatus R. antechini*’, which has been detected in *I. antechini* ticks¹⁷⁴, “*Candidatus R. tasmanensis*” detected in *I. tasmani* ticks¹²⁰, “*Candidatus R. argasii*” from *Argas dewae* ticks (Chapter 5), a *Rickettsia* detected in *I. tasmani* ticks collected from koalas in Port Macquarie²⁶⁵ and another SFG *Rickettsia* detected in *I. tasmani* collected from Tasmanian devils²⁶⁴.

1.7.3.1. Murine typhus (*Rickettsia typhi*)

In 1922 the doctor Frank Hone in Adelaide reported several cases of what he described as a disease ‘closely resembling typhus fever’ also known as ‘wheat disease’ by the local wheat lumpers (dock workers). Sixteen cases were reported with a mortality rate of around 20%. All cases had a rapid onset of symptoms including fever, malaise and a macular rash. He was quick to dismiss the assumption that the patients were suffering typhoid fever, due to the lack of abnormalities in the bowel when performing post mortem autopsies, and a rash

that did not look consistent with typhoid. Weil-Felix test results showed agglutination with the OX19 strain, which was consistent with a rickettsial infection (not scrub typhus). This was the first report of *R. typhi* (the agent of murine typhus) infection in Australia¹⁰⁸. A year later, Hone reported 21 more cases of disease 'closely resembling typhus fever'. He noted that unlike the previous cases that had occurred around Port Adelaide, a number of these new cases occurred throughout Adelaide and its suburbs. He also noted that the cases were distributed evenly throughout the year with a slightly higher prevalence in Autumn¹⁰⁹.

In 1926, Wheatland described thirty eight cases as '(resembling) very closely' the symptoms reported by Hone in Adelaide, with fever, headache and a macular rash of the body and limbs, although with only a 3% mortality rate²⁷⁹. Locals referred to the disease as 'mouse fever' as it usually occurred during mouse plagues, where thousands of mice would swarm on farms in the area²⁷⁹. Wheatland concluded that the disease was likely the same as Hone had described in Adelaide, that it was not contagious and that it was most likely transmitted by an 'ecto-parasite associated with (mice)'²⁷⁹.

In 1927, Hone published an update on murine typhus in Adelaide, reporting an additional 85 cases with 'scarcely a month (passing) without one or more of these cases being under observation somewhere in or about Adelaide'¹¹⁰. He also confirmed Wheatland's assumptions that the disease was associated with rats or mice or their arthropod vectors (primarily rat fleas), although he suspected infection by inhalation rather than a bite, noting that the 'disturbance

of material and of rodent population also plays an important part in production of fresh cases'¹¹⁰.

In 1930 the first two cases of murine typhus in New South Wales were reported¹⁶³, followed by a third case in 1936²⁴⁷. A retrospective study published in 1936 reported an additional 29 cases in New South Wales from 1927 to 1935 and included the first reported fatal case of murine typhus in the state¹¹⁴. Over the next 18 years several more cases from Queensland^{152, 153}, South Australia⁶⁸ and a questionable case from Melbourne, (possibly contracted in Queensland) were reported¹⁴¹.

In 1954 the first report of murine typhus from Western Australia was published. This study reported 1332 cases of murine typhus with a mortality rate of 3.7% occurring between 1927 and 1952, primarily in the city of Perth and surrounding suburbs²²⁰. Saint demonstrated seasonal fluctuations in the number of cases and noted that the common denominator with all of the cases was exposure to, and inhalation of dust from rat-infested areas rather than contact with rats and mice or their ectoparasites²²⁰.

After a report of murine typhus following a mouse plague in 1960⁵⁸, there was a lapse in reported cases until 1991 when Forbes published a case study of a 41 year old male from Queensland, who was only diagnosed with a rickettsial infection as an afterthought when other possibilities were exhausted. The author questioned the number of cases of rickettsial infection that go undiagnosed due to a lack of awareness and testing of the disease in Australia⁸⁰. Over the next few years several other cases were reported in Queensland⁹⁶ and Western

Australia^{15, 130, 165} and in 2004 the first confirmed Victorian case of murine typhus was reported¹²³.

1.7.3.2. Queensland tick typhus (*Rickettsia australis*)

In 1946 Brody reported the case of a 50 year old female who developed fever, headache and a rash several days after collecting wood from scrub land near Gordonvale in North Queensland. It was noted that her symptoms were milder than was usual for scrub typhus infection in the area³⁰. Serology results using the Weil-Felix test returned positive agglutination for both the OX2 and OX19 strains, which was consistent with a SFG rickettsial infection. However up to this point only TG (*R. typhi*) and scrub typhus group (STG) (*O. tsutsugamushi*) *Rickettsia* were known to be present in Queensland³⁰.

Later the same year a second report of 12 cases of suspected tick typhus were reported from around the Atherton Tableland in North Queensland. As with a number of the scrub typhus cases reported at the time, the subjects were soldiers training in the jungles of northern Queensland⁹. All 12 patients were tested using the Weil-Felix test and returned positive agglutination for the both the OX2 and OX19 strains, confirming a SFG infection, which Andrew tentatively named 'North Queensland tick typhus'⁹. This was later amended to 'Queensland tick typhus' (QTT) after it was noted that the disease was not confined to northern Queensland⁸. Blood from the 12 patients was inoculated into guinea pigs and from this both the PHS and FIK strains were isolated⁹. The same year a further study comparing both the PHS and FIK strains with the agent of murine typhus (*R. typhi*) found that QTT was not caused by *R. typhi*. It also mentioned *Ixodes holocyclus* as a possible vector⁸⁶. Later the same year a

third article was published that confirmed the PHS strain as a novel rickettsial species, most likely of the SFG¹⁹².

In 1948 three cases of QTT were reported from southern Queensland. All three cases had similar symptoms to those mentioned before with fever, headache, macular rash and malaise. The Weil-Felix tests confirmed SFG infection, and *Ixodes holocyclus* was again the suspected vector²⁴³. In 1950, the name *Rickettsia australis* was proposed for the aetiological agent of QTT^{186, 187}.

In 1954, a sixteen year old youth from Mount Tamborine in southern Queensland was admitted to Brisbane hospital suffering fever, headache and a rash several days after discovering a tick attached to his scalp. Weil-Felix tests confirmed a SFG rickettsial infection¹⁶². A SFG rickettsia was isolated from his blood by a series of passages into mice and this isolate was named the J.C. strain¹⁹³. Serological testing showed both the J.C. and PHS strains to be serologically identical¹⁴⁶.

Ixodes holocyclus was often considered to be the vector for QTT⁶⁰ but it was not until 1974 that *R. australis* was successfully isolated from both *I. holocyclus* and *I. tasmani*⁴¹.

In 1979, the first non-Queensland case of QTT was reported from Sydney⁴⁰, followed by several other reports from, Sydney^{111, 112, 270}, Queensland^{10, 191, 213, 228}, and Victoria⁶⁷. There have, however, been only two confirmed deaths due to QTT^{191, 228} and it is typically considered to be a mild rickettsial illness¹⁵⁴. In contrast, three cases from northern Queensland were shown to be quite severe with symptoms including renal failure, severe pneumonia and digital gangrene¹⁵⁴.

1.7.3.3. Flinders island spotted fever (*R. honei*)

In 1991 Flinders Island, located in the Bass Strait between Tasmania and Victoria, was reported as a new endemic focus for SFG rickettsia in Australia. With a population of around 1000 people the island had only one medical practitioner²⁴². Over the 17 year period that he worked on Flinders Island he noticed many patients presenting with fever and a rash, with 26 cases in particular that all had very similar clinical manifestations, such as fever, headache, myalgia, arthralgia and a maculopapular rash²⁴².

IFA was performed on sera from all 26 patients and 335 healthy individuals and the Weil-Felix test was performed on 11 of the 26 patients. Of the 26 samples tested with IFA, 20 (77%) were positive for SFG rickettsia, with only 1% of the healthy population testing positive for SFG antibodies. With the Weil-Felix test 4 of the 11 sera (36%) reacted with the OX19 and OX2 antigens confirming SFG infection, which was named Flinders Island spotted fever (FISF)⁹⁷.

Between 1987 and 1990, twenty four cases of suspected FISF from Victoria, Flinders Island and mainland Tasmania were tested for the presence of SFG antibodies. Four of the patients were sero-positive and the other 20 subsequently sero-converted. The blood of 14 of the patients was inoculated into guinea pigs, rats and neonatal mice. From the 14 patient samples, two isolates of the rickettsial agent were obtained⁹⁸. This rickettsia was thus confirmed as the causative agent for these two cases.

At the same time numerous vertebrate and invertebrate specimens (mainly ticks) were tested from the same regions. Twenty two percent of the vertebrates but none of the invertebrate specimens from Flinders Island were

positive for SFG rickettsia, whereas 84% of vertebrate samples and two pools of invertebrate specimens from Gippsland were positive⁹⁸. The two FISF isolates obtained were compared to *R. australis* by sequencing and southern blotting techniques and it was found that the two differed sufficiently to suggest that the FISF agent may be a novel *Rickettsia* species¹¹. Further genetic¹² and monoclonal antibody²³⁹ comparisons confirmed that it was a new species of SFG rickettsia and in 1998 it was officially named *R. honei*²⁴⁰. In 2003 the vector for FISF was proven to be *Bothriocroton* (previously *Aponomma*) *hydrosauri*, a reptile tick, commonly affecting blue-tongue lizards (*Tiliqua* spp.)²⁸⁰.

1.7.3.4. Australian spotted fever (*R. honei* strain *marmionii*)

Between 2001 and 2003 four cases of rickettsial infection were detected near Adelaide in South Australia. All four had symptoms consistent with a SFG infection including fevers, rigor, malaise and a macular rash, although one patient's symptoms were milder. In two of the four patients, there was a clear rise in antibody levels to SFG rickettsia. Rickettsial DNA was detected in two of the patients, including the patient who had milder symptoms and no antibodies present during the acute phase. Sequencing of the rickettsial DNA identified the agent as having the closest phylogenetic similarity to *R. honei* (Flinders Island spotted fever)⁶⁹. Up to this time, FISF had not been detected elsewhere, other than Flinders Island²⁴².

The same year as the first case was published a *Haemaphysalis novaeguineae* tick was removed from a male subject in Queensland. A week later he

developed symptoms consistent with a SFG infection including fever, muscle aches, malaise and a spotted rash. Rickettsial DNA was detected in the tick and sequencing identified it as having the closest phylogenetic similarity to *R. honei*¹³⁹.

While the principal reservoir for FISF on Flinders Island is the reptile tick *Bothriocroton hydrosauri*²³⁷ this *R. honei*-like *Rickettsia* was detected in *H. novaeguineae* ticks, which are typically associated with mammals in Australia²¹².

A third article from 2005 reported three cases of FISF, with two from South Australia and the third from Schouten Island off the east coast of Tasmania. All three had symptoms consistent with FISF and rickettsial DNA was detected in all three samples, with the closest homology to *R. honei*²⁶⁰. The causative agent for the previous mentioned cases was identified in 2007 as being *Rickettsia honei* strain marmionii, and this was defined as the agent of Australian spotted fever²⁵⁹. This article also identified seven additional cases of Australian spotted fever (ASF), with five in Queensland, one in South Australia and one in Tasmania.

In 2008 ASF was shown to be associated with 14 cases of chronic fatigue syndrome from Victoria, with the detection of rickettsial DNA in peripheral blood mononuclear cells (PBMC). The agent was isolated from the blood of two patients, but was not successfully maintained in culture²⁵⁷.

1.7.4. *Orientia*

1.7.4.1. Scrub typhus (*Orientia tsutsugamushi*)

A report of various diseases with fever was published in Australia in 1934¹⁵¹. This review included 'Mossman fever', 'coastal fever' and 'scrub fever', mentioning cases dating back to 1910, although no conclusions were drawn on the cause of these diseases. In 1935 a report on the reclassification of 'Mossman' and 'coastal fever' was also the first confirmed report of scrub typhus in Australia¹⁴⁰. It had been noted that the first settlers of the region in 1877 suffered outbreaks of fever, and that the fevers were known to the local aboriginals well before the arrival of European settlers¹⁰⁴. Symptoms varied with these diseases but in a number of cases the patients had fever, muscle aches and a rash. Symptoms lasted for around 14 days and results from the Weil-Felix test indicated *Orientia* (then *Rickettsia*) *tsutsugamushi* as the causative agent¹⁴⁰. It is now known that these diseases were probably a mixture of diseases including malaria, leptospirosis, dengue fever and scrub typhus⁹³. Later the same year a second report on 'coastal fever' was published, including eight more cases from northern Queensland, with results consistent with scrub typhus, and suggestions that a mite may be involved in the transmission of the disease²⁶¹.

In 1938 Mathew noted that while some of the cases of endemic typhus in Northern Queensland reacted with the OX-19 strain in the Weil-Felix test, and had symptoms consistent with murine typhus, there were cases that reacted to only the OXK strain, with eschars on the patients which resembled those of

‘mite borne Japanese river fever’. He also noted that the symptoms were the same as those in patients with scrub typhus¹⁵³.

A article by Heaslip published in 1940 reported a case of ‘coastal fever’ contracted south of Cairns that was in fact scrub typhus and noted that ‘coastal fever’ was caused by many different diseases¹⁰³. Heaslip later reported the similarities between the earlier reports of coastal and Mossman fever and Japanese river fever and reported a number of cases of scrub typhus in Queensland. He also suggested that transmission of the disease was via a mite vector¹⁰⁴.

With the beginning of the 2nd world war, areas of bushland and jungle in northern Queensland were used for military training exercises²³⁴. This resulted in a marked increase in scrub typhus cases, typically involving soldiers^{7, 46, 234}. This influx of cases, combined with the accurately recorded locations and times of exposure, allowed researchers to learn a great deal about the characteristics of Australian scrub typhus.

Over the next 40 years numerous cases of scrub typhus in Queensland were reported^{41, 57, 59, 155, 230}, and it was believed this was the only area in Australia where scrub typhus occurred, although in 1961 Derrick speculated that the range of scrub typhus may be broader⁵⁷. In 1991 a article was published reporting two cases of scrub typhus from the Northern Territory⁵². The two near fatal cases were contracted in Litchfield National Park, a recently opened park allowing access to rainforest areas⁵². Up to this point, Northern Territory was not considered to be a focus for scrub typhus or *Leptotrombidium deliense*, the vector for the disease, although a study of Litchfield National Park in 1993

reported the presence of *L. deliense* on rats²⁰. In the same year five additional cases of scrub typhus were reported from the same region⁵¹. The first, and so far only fatal, Australian case of scrub typhus from Litchfield National Park occurred in 1996⁵⁰. The Litchfield strain of *O. tsutsugamushi* was isolated from a patient in 1999, and it was found to be serologically divergent from other known strains. It was named *O. tsutsugamushi* strain Litchfield¹⁶⁶. Up to this point, all reported cases of scrub typhus in the Northern Territory had been contracted in Litchfield National Park. In 2004 however, a 40 year old male contracted scrub typhus while visiting a patch of rainforest 20km east of Litchfield National Park²⁰⁰, so the geographical boundaries may extend well beyond this park.

The range of scrub typhus was broadened even further when, after visiting the Kimberley region in Western Australia, a 19 year old engineering student developed rigors, sweats, myalgia and a fever. The presence of a scrub typhus infection was serologically confirmed and the symptoms subsided when he was treated with appropriate antibiotics¹⁹⁷. A serological survey of 920 patients for the presence of SFG, TG and STG *Rickettsia* in northern Western Australia confirmed the presence of scrub typhus in the state with 2.6% of the sera having antibodies to *O. tsutsugamushi*⁹⁴.

1.7.5. This Study

Numerous studies over the years have given an insight into the species and epidemiology of Rickettsiales and rickettsial diseases in Australia. However, significantly more research is still required to elucidate the full picture. This

thesis contains several studies that add to the knowledge of epidemiology and diversity of *Rickettsiales* in Australia.

Chapter 2. Materials and Methods

2.1. Rickettsial Isolation

2.1.1. Blood sample processing

Blood was collected in EDTA tubes to prevent clotting. The tubes were centrifuged at 6000xg using a 3K15 centrifuge (Sigma Laboratory Centrifuges, Germany) to separate packed red blood cells (RBC), the buffy coat (white blood cells) and plasma. The buffy coat was collected using a plastic transfer pipette (Samco, USA) and placed in a 10ml centrifuge tube (Techno-plas, Australia) containing Puregene RBC lysis solution (Gentra Systems, USA). This mix was incubated at 37°C for 10 minutes for red blood cell lysis, after which it was centrifuged again at 6000xg for 10 minutes. The buffy coat pellet was rinsed twice with phosphate buffered saline (PBS) and resuspended in approximately 300µl of PBS. The buffy coat mix was then divided, with half being placed in cell culture for growth and isolation of the rickettsial agent, and the rest subjected to DNA extraction for real-time polymerase chain reaction (qPCR) detection and subsequent sequencing.

2.1.2. Tick sample processing

Ticks were collected by removing them from the host with forceps, and placing them in a humid container. Once at the laboratory, they were washed first with 70% ethanol for 30 seconds to kill any surface bacteria. As the *Rickettsia* are an internal organism they were unharmed.

The ticks were then rinsed with sterile PBS for a further 30 seconds to remove any residual ethanol that may affect the *Rickettsia* once they were exposed.

After washing the ticks, they were placed in a 1.5ml centrifuge tube (SSI, Australia) in 400µl of PBS. They were then homogenised using disposable centrifuge pestles (Edwards Instruments Co., Australia).

The crushed tick mix was then filtered using a 0.45µm sterile syringe filter (Millex, USA), to remove the tick debris and any other larger bacteria. *Rickettsia* are small enough to pass through this filter. As with the blood sample preparation, the filtrate was divided, with half being put into culture and the other half subjected to DNA extraction for qPCR and sequencing.

2.1.3. Flea sample processing

Fleas were collected using a commercial flea comb. Animals were brushed with the flea comb and any fleas removed were placed in a humid container. Once at the laboratory, the fleas were separated into batches of 5 to 10 fleas and then processed using the same protocol used for the tick samples in section 2.1.2.

2.2. Cell Culture

Cell cultures were derived from a number of sources, four of which formed a monolayer and three which did not. The four that formed a monolayer were derived from Green Monkey kidney cells (Vero), South African Clawed Toad epithelial cells (XTC-2), Mouse Fibroblasts (L929) and dog macrophage cells (DH-82). The three which remained in suspension were derived from Human Promyelocytic Leukaemia cells (HL-60) and Human Megakaryocytic Leukaemia cells (DAMI and HI-MEG).

All cell lines except XTC-2 were grown at 35°C in 25cm² cell culture flasks (Iwaki, Canada). The cell culture media for growing the cells was RPMI 1640 medium (Gibco, Australia), supplemented with 25mM HEPES (Gibco, Australia), 200mM L-Glutamine (Gibco, Australia) and 5% newborn calf serum (Gibco, Australia). The XTC-2 cells were grown at 28°C in 25cm² cell culture flasks. The media used was Leibovitz L-15 (Gibco, Australia) supplemented with 200mM L-Glutamine (Gibco, Australia), 0.4% tryptose phosphate broth (Oxoid, Australia) and 10% newborn calf serum (Gibco, Australia).

The cell lines were examined daily under a light microscope to determine their confluence. Once confluent, the cell lines were inoculated with material suspected of containing rickettsiae.

2.3. Freezing Samples

Rickettsial cultures were frozen as viable stock for later use. This procedure involved first using a cell scraper (TPP, Switzerland) to lift the cell monolayer. The cell-medium mix was transferred into a 10ml centrifuge tube and centrifuged at 6000xg for 10 minutes using a 3K15 centrifuge. The supernatant was removed and the pellet was resuspended in cell culture freezing media (CCFM) (Gibco, Australia). This mix was then aliquoted into 2ml plastic cryogenic vials (Iwaki, Canada) and stored in liquid nitrogen.

2.4. Molecular Methods

2.4.1. DNA sample preparation

DNA was extracted using either the QIAmp DNA Blood Mini Kit (Qiagen, Germany) or the Real Genomics™ Gene DNA extraction kit (Real Biotechnology Corporation, Taiwan). A 200µl sample was used for the Qiagen kit while 100µl was used for the Real Genomics kit. Extractions were performed as per the manufacturer's instructions. The extracted DNA was eluted into 50-200µl of the elution buffer supplied in the kit.

2.4.2. Primer/probe design and validation

2.4.2.1. Primer/probe set design

The first step of primer or probe design involved selecting the target gene and organism(s). The *rrs*, *gltA*, *rOmp-A*, *rOmp-B*, *sca4* and *orf17* genes were targeted for *Rickettsia* spp. While the *rrs* and *gltA* genes were targeted for *Anaplasma* spp. and *Ehrlichia* spp. For *Orientia* spp., the *110kDa*, *56kDa* and *47kDa* genes were the targets for primer/probe design. Three representative sequences of the target gene for each organism were obtained using the BLAST program (National Center for Biotechnology Information, USA). The sequences were aligned using "MegAlign", which is part of the DNA Star package (DNASTAR Inc., USA), to obtain a consensus sequence.

The consensus sequence was entered into the AlleleID software package (Premier Biosoft, USA). This software produced a list of primers and probes that targeted the consensus sequence entered. They were sorted using a scoring

system that took into consideration such parameters as melting temperature, primer dimers, and GC grouping within the primer and probe sequence.

The primer/probe set with the highest score was selected and aligned against the set of sequences within “MegAlign” to check that it aligned with all targets. If this set was unsuccessful the next one on the list was selected and tested. This continued until a successful set was obtained.

The primers were purchased from Invitrogen (Australia), and the probes from Biosearch Technologies (USA). The assay was optimised by running multiple reactions with varying $MgCl_2$ and primer/probe concentrations. The combination of concentrations that obtained the lowest C_t using the least amount of reagents was chosen for future assays.

2.4.2.2. Testing Sensitivity

The primer set’s target region was amplified using the optimised PCR minus the probe. This PCR product was inserted into a pCR[®] 2.1 Plasmid (Invitrogen, Australia), which was subsequently transformed into One Shot[®] Top10 chemically competent *Escherichia coli* cells (Invitrogen, Australia) using the TA Cloning Kit (Invitrogen, Australia) manufacturer’s instructions. After subsequent selection and growth of a positive clone, it was pelleted and the plasmid was extracted using a QuickLyse Mini Prep kit (Qiagen, Germany). The plasmid concentration was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, USA), and from this the theoretical number of copies was mathematically obtained (see section 10.1).

Next, a titration was prepared using a series of 10-fold dilutions in duplicate to determine a copy number endpoint to the assay and to determine its sensitivity.

2.4.2.1. Testing Specificity

To test the specificity of the assay, DNA was extracted from the target organisms as well as other representative members of the *Rickettsiales* (*Rickettsia* spp., *Anaplasma* spp. *Ehrlichia* spp., and *Orientia* spp.). DNA of other medically important organisms was also extracted (*Bartonella vinsonii*, *Coxiella burnetii*, *Escherichia coli*, *Enterococcus faecalis*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae*).

The qPCR was run using the extracted DNA along with a positive control to test if it amplified any organisms other than those intended. Each DNA sample was also tested using a universal 16S conventional PCR described in section 2.4.4. to confirm the presence of bacterial DNA.

2.4.3. qPCR detection

When performing a qPCR reaction two different mastermixes were prepared depending on the reaction to be performed. Each mastermix contained all of the reagents except the DNA sample. The first mix was used if concentrations of greater than 3mM MgCl₂ were needed. This mix contained 2XPlatinum[®] qPCR SuperMix-UDG Mastermix (Invitrogen, Australia), MgCl₂, forward and reverse primers, and the probe. Primer, probe and MgCl₂ concentrations varied

depending on the specific conditions of each primer/probe set determined in section 2.4.2.1.

The second mastermix was used when concentrations of less than 3mM MgCl_2 were required and contained 10X PCR reaction buffer (Invitrogen, Australia), MgCl_2 (Invitrogen, Australia), forward and reverse primers, probe, dNTPs (Invitrogen, Australia) and Taq DNA polymerase (Invitrogen, Australia). As with the first mix; primer, probe and MgCl_2 concentrations varied depending on the specific conditions determined in section 2.4.2.1.

After the mastermix was prepared it was aliquoted into each reaction tube along with the specific DNA samples. A known positive sample was added to one tube as a positive control and water was added to a second tube as a 'no template control' (NTC).

The qPCR reaction was performed in a Rotor-Gene 3000 (Corbett Research, Australia), with an initial 3 minute 50°C incubation to allow the uracil DNA glycosylase (UDG) to digest any PCR template contamination, followed by a 95°C incubation to activate the Taq DNA polymerase. After this the temperature was cycled 40-60 times; first with a 95°C denaturation step, followed by a low annealing temperature of between 40-60°C depending on the specific primer/probe set used. A few primer/probe sets also required a further 72°C extension step in the cycle. The level of fluorescence for each tube was detected and recorded after every cycle with the results being displayed graphically.

2.4.4. Conventional PCR

Conventional PCR served two purposes; the first was to detect the presence of known rickettsial pathogens in clinical and environmental samples. This was achieved by designing primers that target sections of a gene conserved within a particular species, genus etc. The formation of a band located at the correct base pair size, when compared with the DNA ladder, confirmed the presence of the organism(s).

The second purpose involved amplifying and sequencing targeted genes in order to identify unknown organisms. Primers used targeted various genes of the *Rickettsiales* including the *rrs*, *gltA*, *rompA*, *rompB*, *sca4*, and *orf17* for SFG *Rickettsia*, *tsa56*, *rompB* and *rrs* for STG *Rickettsia*, and *gltA* and *rrs* for *Anaplasmataceae* (Table 3). All primer sets used the same mastermix consisting of 10X PCR reaction buffer, MgCl₂, forward and reverse primers, dNTPs and Taq DNA polymerase. The primers, MgCl₂ concentrations and annealing temperatures varied with each set.

A Rotor-Gene 3000 or a Progene (Techne, United Kingdom) was used to run the reactions.

The sample was initially incubated at 95°C to activate the Taq DNA polymerase. It was then cycled 40-50 times; first with a 95°C denaturation step, followed by a low annealing temperature between 40-60°C (shown in Table 3) and finally at 72°C to allow the complete extension of the fragment.

Once the assay had been completed, the PCR product was loaded onto a 1% agarose gel (Amersco, USA) containing SYBR Safe DNA gel stain (Invitrogen,

Australia). A 100 bp DNA ladder (Invitrogen, Australia) was also added to a well to allow post run band size determination.

The gel was immersed in 1x TAE buffer (Amresco, USA) and a voltage was applied across it using a Power Pac 300 (Bio-Rad, USA) power supply to separate the different size DNA bands. The gel was then viewed on a Safe Imager™ blue light transilluminator (Invitrogen, Australia).

Table 3. Name, conditions and literature references for oligonucleotides used for conventional PCR.

Target gene	Primer name	Primer sequence (5'→3')	MgCl ₂ (mM)	Annealing temperature (°C)	Reference
<i>orf17</i> (17kD gene)	MTO-1	GCT CTT GCA ACT CTA TGT T	2	51	274
	MTO-2	CAT TGT TCG TCA GGT TGG CG			
<i>gltA</i> (Citrate Synthase)	CS-162-F	GCA AGT ATC GGT GAG GAT GTA ATC	3	48	240
	CS-398-SF	ATT ATG CTT GCG GCT GTC GG			
	CS-731-SR	AAG CAA AAG GGT TAG CTC C			
	RpCS877n	GGG GAC CTG CTC ACG GCG G			
	RpCS1258p	ATT GCA AAA AGT ACA GTG AAC A			
<i>rrs</i> (16S rRNA gene)	rRNA1	AGA GTT TGA TCC TGG CTC AG	2	51	214
	rRNA2	AAG GAG GTG ATC CAG CCG CA			
	rRNA3	CCC TCA ATT CCT TTG AGT TT			
	rRNA4	CAG CAG CCG CCG TAA TAC			
<i>rompA</i> (rickettsial OmpA)	Rr190.70p	ATG GCG AAT ATT TCT CCA AAA	1	48	206
	Rr190.602n	AGT GCA GCA TTC GCT CCC CCT			
<i>rompB</i> (rickettsial OmpB)	120-M59	CCG CAG GGT TGG TAA TGC	2	51	217
	120-807	CCT TTT AGA TTA CCG CCT AA			
	120-607	AAT ATC GGT GAC GGT CAA GG			
	120-1497	CCT ATA TCG CCG GTA ATT			
	120-1378	TAA ACT TGC TGA CCG TAC AG			
	120-2399	CTT GTT TGT TTA ATG TTA CCG T			
	120-2113	CGA TGC TAA CGT AGG TTC TT			
	120-2988	CCG GCT ATA CCG CCT GTA GT			
	120-2788	AAA CAA TAA TCA AGG TAC TGT			
	120-3599	TAC TTC CCG TTA CAG CAA AGT			

Table 3 continued.

Target gene	Primer name	Primer sequence (5'→3')	MgCl ₂ (mM)	Annealing temperature (°C)	Reference
<i>sca4</i> (gene D)	D1f	ATG AGT AAA GAC GGT AAC CT	2	51	223
	D928r	AAG CTA TTG CGT CAT CTC CG			
	D767f	CGA TGG TAG CAT TAA AAG CT			
	D1390r	CTT GCT TTT CAG CAA TAT CAC			
	D1219f	CCA AAT CTT CTT AAT ACA GC			
	D1876r	TAG TTT GTT CTG CCA TAA TC			
	D1738f	GTA TCT GAA TTA AGC AAT GCG			
	D2482r	CTA TAA CAG GAT TAA CAG CG			
	D2338f	GAT GCA GCG AGT GAG GCA GC			
	D3069r	TCA GCG TTG TGG AGG GGA AG		48	

2.4.5. Sequencing

Overlapping primer sequences, previously published (table 3), were used to amplify the fragments of each gene. These specific DNA fragments were cloned and the DNA concentration was determined using the methods in section 2.4.2.2. A Big Dye terminator reaction was then performed, which involved adding approximately 250ng of cloned DNA into a mastermix containing Big Dye terminator, 5x dilution buffer, primer and water. Using a Progene or GeneAmp PCR System 2400 thermocycler (Applied Biosystems, USA) the mix was first heated to 96°C for 1 minute. It was then cycled 26 times with an initial heating for 10 seconds at 96°C, followed by annealing at 50°C for 5 seconds and finally extension at 60°C for 4 minutes.

After the Big Dye reaction was completed, the sample was washed with an EDTA, sodium acetate and ethanol mix then a second time in 70% ethanol. It was finally dried and sent to the Australian Genomic Research Facility (AGRF), where it's sequence was determined using an ABI Prism 3730xl DNA Analyser (Applied Biosystems, USA).

The sequence was then analysed using bioinformatics.

2.4.6. Bioinformatics

The sequence data was assembled and edited using the SeqMan Pro program within the Lasergene® software package (DNASTAR, Inc., USA), and the identity of the organism was determined using the BLAST analysis software on the NCBI website. Using the neighbour-joining and maximum-parsimony methods within the MEGA 4 software package²⁴⁵ and the maximum-likelihood

method within the PHYLIP software package⁷⁵, phylogenetic trees were created. These trees displayed the percentage phylogenetic divergence of each sequence, represented by the branch length, and the stability of each branch, numerically represented by bootstrap values displayed at each node.

2.5. Serology

2.5.1. Microimmunofluorescence

Microimmunofluorescence assays (IFA) were performed on various serum samples to test for the presence of antibodies to SFG or STG rickettsia. This involved applying an antigen consisting of a mixture of SFG rickettsiae (*Rickettsia akari*, *R. australis*, *R. conorii*, *R. honei*, *R. rickettsii* and *R. siberica*) or for the STG slides a STG antigen (consisting of *O. tsutsugamushi* stains Kato, Karp or Gilliam) onto 40 well slides and fixing with absolute acetone. A titration of the sera samples was prepared by a series of dilutions in a 2% casein/phosphate buffered saline (PBS) solution and these were then incubated with the antigens at 37°C for 30 min followed by a wash step of 3 x 5 min in PBS. A 1:50 diluted fluorescein isothiocyanate (FITC) labelled goat anti-dog immunoglobulin G (IgG) antibody (Kirkegaard & Perry Laboratories, USA) for the dog sera, FITC labelled goat anti-cat IgG antibody (Kirkegaard & Perry Laboratories, USA) for the cat sera or FITC labelled goat anti-human IgG antibody (Kirkegaard & Perry Laboratories, USA) for human sera was then added to the slides and incubated at 37°C for 30 min before being washed 3 x 5 min in PBS. Results were obtained by viewing slides using a Leica DM LS microscope (Leica, Germany) with an ultraviolet fluorescence illuminator.

All samples were screened at a predetermined level and any positive samples were titrated until an end point was determined.

Chapter 3. A serological prevalence study for rickettsial exposure of cats and dogs in Launceston, Tasmania, Australia

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3.1. Abstract

A sero-epidemiological study of cats and dogs in the Launceston area of Tasmania, Australia was undertaken to determine the prevalence of antibodies to Spotted Fever Group (SFG) rickettsia. Results showed that 59% of cats and 57% of dogs were positive for antibodies to SFG rickettsia. There was however,

no correlation between the animals' health and seropositivity at the time of testing, suggesting that rickettsial exposure was unrelated to the ill-health of these two domestic animal species.

3.2. Introduction

Rickettsiae are obligate intracellular coccobacilli bacteria of the α -proteobacteria class²⁶⁷. The genus *Rickettsia* is molecularly divided into 2 groups; the typhus group (TG) and the spotted fever group (SFG)²⁰³. With the exception of *Rickettsia felis* and *R. akari*, SFG rickettsia are transmitted by tick vectors, which are also the natural reservoir of these bacteria²⁰³.

SFG rickettsia cause a number of human diseases in Australia, including Queensland Tick Typhus (*R. australis*)⁹, Flinders Island Spotted Fever (FISF) (*R. honei*)²⁴², and more recently, a variant form of FISF (*R. honei* strain *marmionii*)²⁵⁹. These three diseases have similar symptoms with malaise, headaches, chills, fever, and rash, and all are treatable with appropriate antibiotics^{181, 186, 240}. Additionally, *R. honei* strain *marmionii* has been isolated from patients with chronic illness including fatigue²⁵⁷.

There have been only three published cases of human SFG rickettsia disease on mainland Tasmania^{42, 259, 273}, although there have been a number of published reports of disease from surrounding islands^{97, 98, 242}. *Rickettsia honei* has been isolated from the tick species *Bothriocroton* (formally *Aponomma*) *hydrosauri* on Flinders Island²³⁷. *Bothriocroton hydrosauri* is found in most south-eastern areas of Australia³⁷ where human cases of FISF are known to occur²⁶⁰.

The study was focused around Launceston, which is the second largest city in Tasmania. Over 63,000 people live in Launceston itself, and approximately 100,000 including the surrounding area³⁹. As well as significant areas of farming land, Launceston is surrounded by eucalypt forest with medium to dense undergrowth that supports a wide variety of fauna, making it a suitable tick habitat. The magnitude of the domestic dog and cat population is unknown.

Determining the seroprevalence of SFG antibodies in domestic dogs and cats can indirectly indicate the presence of a SFG rickettsia within a particular region^{142, 226}.

3.3. Materials and methods

3.3.1. Sample and data collection

Sera from 368 dogs and 150 cats were collected from a veterinary hospital in Launceston, Tasmania over a 16 month period in 2004 and 2005. All tested dogs and cats resided within a fifty kilometre radius of the Launceston town centre.

Information was collected on each animal including, age, sex, geographical origin and clinical state (sick/healthy). Sickness was determined by the presence of one or more clinical markers including fever, weight loss, lameness, aesthenia and swollen lymph nodes²³³.

3.3.2. Detection of antibodies to Spotted Fever Group Rickettsia

Each serum sample was tested for the presence of antibodies to the SFG rickettsia using a microimmunofluorescence assay (IFA)⁹⁷ as described in **section 2.5.1**. Positive and negative cat and dog sera were included in each assay. Control dog sera were obtained from a previous study²²⁶. The cat control sera were supplied by Dr. Helen Owen, University of Queensland, Brisbane, Queensland.

3.3.3. Statistical analysis of serological results

Statistical analysis was undertaken to determine if there was any correlation between the animal's clinical state and rickettsial sero-positivity at 3 different serological "cut-off" values (1/50, 1/100 and 1/200). Chi-square analysis was used to determine P values (Table 4) with P values of less than 0.05 considered statistically significant.

3.4. Results

3.4.1. Serology Results

The results were analysed using three different serum dilution cut-off points to test for any correlation between level of rickettsial seropositivity and current sickness in the animals (Table 4).

At the three defined cut-off points, the dog total sero-positivity was 57%, 32% and 13% at 1/50, 1/100 and 1/200 respectively, while the cats total sero-positivity was 59%, 43% and 29% at 1/50, 1/100 and 1/200 respectively.

Overall, 53% of the dogs and 63% of the cats tested were defined as sick (Table 4).

3.4.2. Statistical analysis

P values were determined for each 2 x 2 cell to determine whether there was a statistically significant correlation between the degree of sero-positivity and animal sickness. Using a level of significance of $P < 0.05$, none of the cells showed statistical significance (Table 4). There was no correlation between dogs and cats being seropositive for SFG rickettsia at the three end points selected and being unwell at the time of serum sampling.

Table 4. Seropositivity for Spotted Fever Group rickettsiae in dog and cat serum tested at 1/50, 1/100 and 1/200 dilutions showing a lack of statistical relationship ($p>0.05$) between clinically sick animals and seropositive animals.

(Seroreactivity at 1/50 dilution)

	Seropositive animals		Seronegative animals		Total		% Positive		P-Value	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Clinically Sick	106	53	88	42	194	95	55%	56%	0.44	0.25
Well	102	36	72	19	174	55	59%	65%		
Total	208	89	160	61	368	150	57%	59%		

(Seroreactivity at 1/100 dilution)

	Seropositive animals		Seronegative animals		Total		% Positive		P-Value	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Clinically Sick	56	37	138	58	194	95	29%	39%	0.25	0.23
Well	60	27	114	28	174	55	34%	49%		
Total	116	64	252	86	368	150	32%	43%		

(Seroreactivity at 1/200 dilution)

	Seropositive animals		Seronegative animals		Total		% Positive		P-Value	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Clinically Sick	23	29	171	66	194	95	12%	31%	0.48	0.51
Well	25	14	149	41	174	55	14%	25%		
Total	48	43	320	107	368	150	13%	29%		

3.5. Discussion

A very high proportion ($>50\%$) of the cats and dogs in the Launceston area had serological evidence of exposure to SFG rickettsia at a 1/50 serum dilution cut-

off. There was however no statistically significant correlation between animal sickness and rickettsial sero-positivity at this or the higher dilutions. This could be due to frequent low level exposure to a SFG rickettsia or exposure to *Rickettsia* that produce mild or no symptoms. Clinical symptoms may have been present earlier however, but were not severe enough to warrant veterinarian care.

A previous study undertaken on the south east coast of Australia found that only 12% of the dogs tested in Tasmania showed exposure to a SFG *Rickettsia*²²⁶. However, our current study represents a much larger sampling pool from a specific area and may display a truer representation of rickettsial exposure in domestic animals in this region of Tasmania.

As our serosurvey involved single sampling of the animals, without convalescent or DNA samples being obtained, we were unable to determine whether the animals were currently infected with a SFG rickettsia or had been exposed in the past.

The results from this study confirm exposure to a SFG rickettsia in dogs and cats on mainland Tasmania. The causative rickettsia and the invertebrate vector for its transmission are still unknown, although the island of Tasmania has a number of species of ticks that would be suitable vectors for SFG rickettsiae.

Ixodes spp. are common and abundant in Tasmania and are known to feed on marsupials and other mammals including dogs, cats, wombats, wallabies and rats. Some (e.g. *I. tasmani*) are also known to bite humans^{98, 212}. The vector for FISF (*Bothriocroton hydrosauri*) is known to bite humans and is found throughout mainland Tasmania²³⁷.

During a 17 year period between 1973 and 1989 there were twenty six reported human cases of “Flinders Island Spotted Fever” (FISF)^{97, 242}. As the entire island had a population of only 1000 people, this was a high incidence of rickettsial infection. Flinders Island is part of Tasmania located 100km off its north east coast. More recently however, FISF has been found to be more widespread throughout the eastern half of Australia^{259, 260}. To date, however, there have only been three published cases of a human SFG rickettsial infection on mainland Tasmania^{42, 259, 273}. This suggests significant under-detection.

We can only hypothesise as to the reason for the rarity of reported human cases of SFG infection in Tasmania. Firstly, the infections may be undiagnosed or misdiagnosed due to a low awareness of the disease in Australia, or the fact that it may only present as a mild infection due to a low virulent strain of rickettsiae.

Secondly, the vector for this *Rickettsia*, which causes sero-positivity in cats and dogs, may not bite humans, and therefore the rickettsial agent is unlikely to pass to a human host. This is less plausible as most ticks that bite other mammals will also bite humans opportunistically.

This study shows that cats and dogs in the Launceston area are exposed (perhaps frequently) to a SFG rickettsia. We do not currently have any comparable data on the level of SFG rickettsia antibodies or the tick bite frequency in the human population within this region.

Future directions for this study could include a sero-epidemiological study of cats and dogs in other parts of Tasmania, which could be expanded to include native animals (e.g. possums, wombats and Tasmanian devils). Blood samples

from these animals could also be used to attempt isolation of the rickettsial agent(s) responsible.

The study could be further expanded to encompass a sero-epidemiology study of the human population of Tasmania, to see whether they also have a high sero-prevalence to SFG rickettsia, as was previously demonstrated in the recognition of FISF⁹⁷.

Chapter 4. *Novel Rickettsia (Candidatus Rickettsia tasmanensis) in Tasmania, Australia*

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4.1. Abstract

A novel rickettsia was detected in *Ixodes tasmani* ticks collected from wild Tasmanian devils. A total of 55% were positive for the citrate synthase gene by quantitative PCR. According to current criteria for rickettsia speciation, this new rickettsia qualifies as *Candidatus Rickettsia tasmanensis*, named after the location of its detection.

4.2. Introduction

Currently in Australia there are 4 known rickettsial species that cause disease in humans of which none have been identified in Tasmania. However there have been three published cases of human rickettsial infection in Tasmania^{42, 259, 273}.

In this study, *I. tasmani* ticks were collected from Tasmanian Devils (*Sarcophilus harrisii*), and these were tested using qPCR and sequencing methods to detect and identify spotted fever group (SFG) rickettsia. Characterization of a novel rickettsial species identified in the ticks was achieved by comparing the sequences of genes⁸¹.

Ixodes tasmani ticks were of particular interest as they are known to be the vector for other rickettsial species in Australia²²⁷ and are also the most common tick species in Tasmania²¹². Additionally they bite humans so are candidates for rickettsial transmission in Tasmania.

Although *Candidatus R. tasmanensis* has not yet been associated with human disease, the possible virulence of this rickettsia cannot be disregarded. In the past there have been examples of rickettsiae initially declared to be non-pathogenic but were later found to be the cause of rickettsial disease in humans. For example *R. parkeri* was discovered in 1939¹⁷⁸ but was only confirmed as a human pathogen in 2004¹⁷⁷.

4.3. Methods

Forty four *I. tasmani* ticks were collected from Tasmanian Devils from various sites between 2005 and 2006, of which thirty six were engorged females, five were nymphs and three were males.

Each tick was processed as described in **section 2.1.2**.

DNA was extracted from the tick homogenates using methods described in **section 2.4.1** and amplification of the *gltA*, *sca4*, *ompA*, and *ompB* genes was performed as described in **section 2.4.4**.

Amplicons were cloned using the methods described in **section 2.4.5** and sequences were assembled, edited and analysed using methods described in **section 2.4.6**. All sequences have been deposited in GenBank (Table 5).

Table 5. GenBank accession numbers of additional sequences used in this study.

Strain	<i>rrs</i>	<i>gltA</i>	<i>rOmpA</i>	<i>rOmpB</i>	<i>sca4</i>
" <i>Rickettsia</i> sp. 518"	-	EU430246	EU430247	EU430242	-
' <i>Candidatus R. tasmanensis</i> ' T120	-	GQ223395	-	GQ223396	GQ223397
' <i>Candidatus R. tasmanensis</i> ' T152-E	-	GQ223391	GQ223392	GQ223393	GQ223394

4.4. Results

Rickettsial DNA was detected in 55% (24/44) of the *I. tasmani* using a *gltA* specific qPCR assay. As the majority of the ticks were engorged females, there was no statistical correlation between the sex of the ticks and presence of rickettsia. The distribution of the ticks collected and degree of positivity are shown in Figure 3.

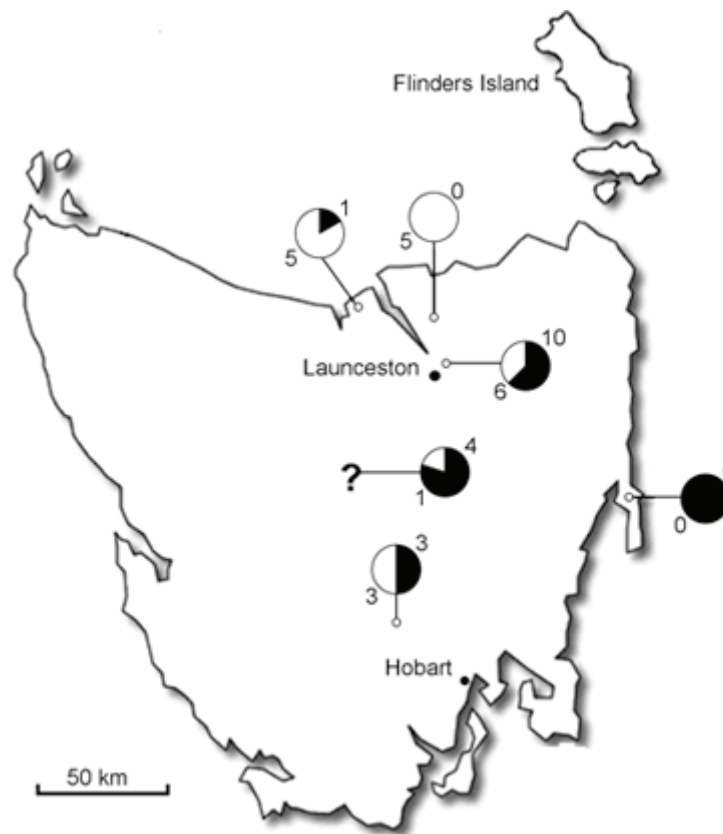


Figure 3. Map of Tasmania, Australia, showing number of positive (black) and negative (white) ticks and their locations. The question mark indicates unknown locations. A total of 55% of the ticks were positive for a spotted fever group rickettsia.

Sequences from the *I. tasmani* ticks were compared to validated sequences¹⁵⁷. The results showed the closest phylogenetic relative for three of the genes as *R. raoultii* strain Khabarovsk, with sequence similarities of 99.1% (1086bp/1096bp), 96.9% (570bp/588bp) and 97.7% (4782bp/4895bp) for the *gltA*, *ompA* and *ompB* genes respectively and 98.1% (2930bp/2988bp) similarity to *R. japonica* strain YM for the *sca4* gene.

Comparison of our sequences with that of a partially sequenced rickettsia (*Candidatus* R. tasmanensis strain T120) previously detected in an *I. tasmani* tick removed from a child near Underwood, Tasmania, (N. Unsworth, PhD thesis, University of Melbourne) found homology levels to be within the species threshold. No data on the clinical state of the child was obtained.

The sequences closely matched genes of a second partially sequenced rickettsia (*Rickettsia* sp. 518) from an *I. tasmani* tick removed from a Tasmanian Devil in Tasmania, by researchers at Macquarie University, New South Wales²⁶³. Of the three partial gene sequences they submitted, *ompB* and *gltA* match to the species level with *Candidatus* R. tasmanensis, but the *ompA* gene however did not. Potentially this could be another new species, although with small fragments sequenced it is difficult to draw conclusions.

The *ompB* gene sequence analysis using the neighbor-joining algorithm is shown in Figure 4. Although all genes were analysed, the *ompB* gene tree was illustrated as it had the strongest bootstrap values and the largest compared fragment size.

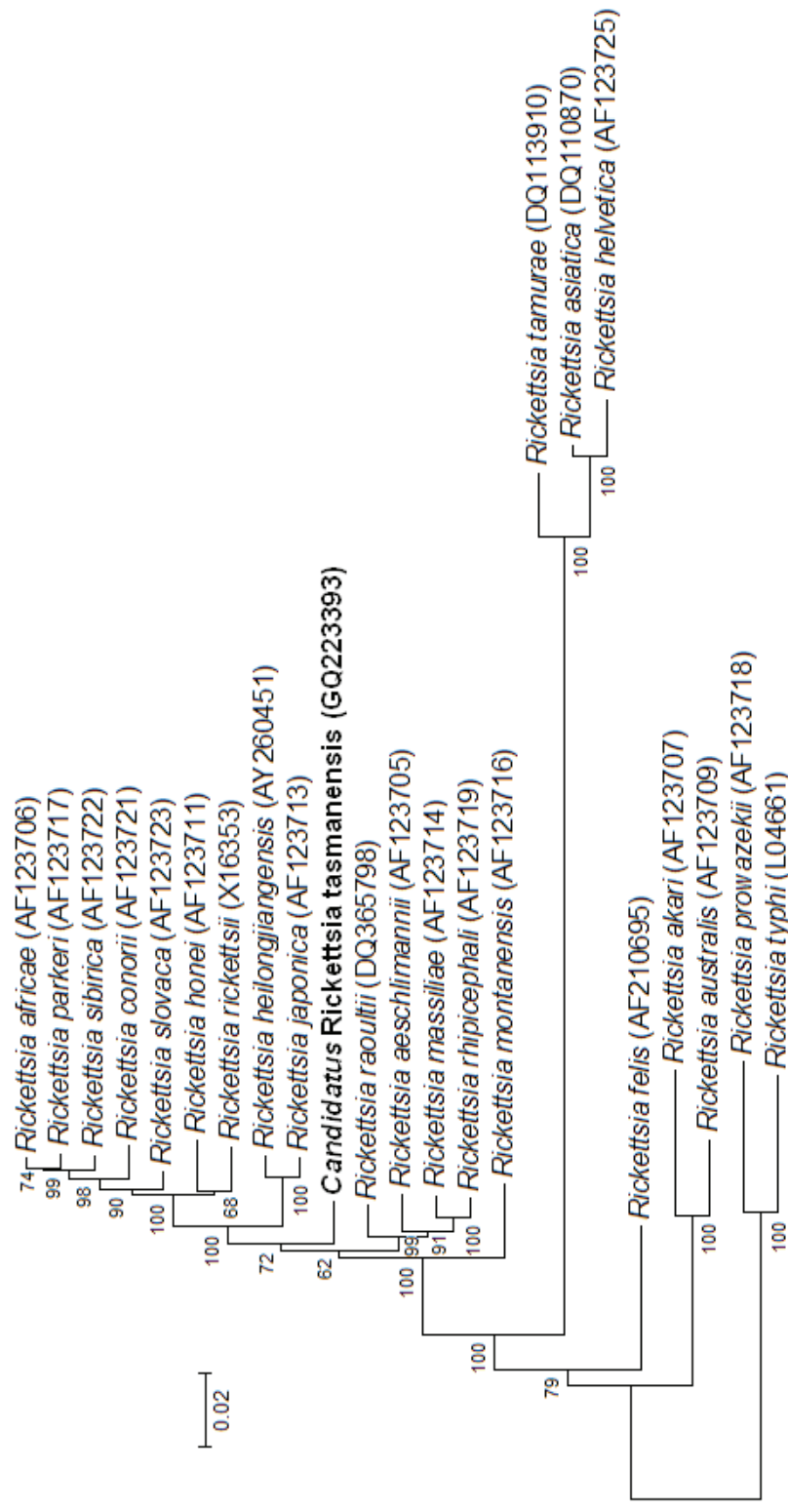


Figure 4. Phylogenetic tree showing the relationship of a 4,834-bp fragment of the outer membrane protein B gene of *Candidatus Rickettsia tasmanensis* (in boldface) compared to all validated rickettsia species. The tree was prepared using the neighbor-joining algorithm within the MEGA 4 software^{24,5}. Bootstrap values are indicated at each node. Scale bar indicates 2% nucleotide divergence.

4.5. Discussion

All 44 ticks were collected from the north eastern and eastern sides of Tasmania. The level of positive samples (55%) contrasts with the small number of reported SFG rickettsial human infections in Tasmania, especially considering the high density of *I. tasmani* in Tasmania, a tick which is known to opportunistically bite humans²¹². This brings up the question; are clinical cases missed because doctors aren't aware of rickettsial disease in Tasmania?

A recent study described previously in the thesis displayed a high level of exposure to a SFG rickettsia in cats and dogs around the city of Launceston, Tasmania¹¹⁹. This study showed high levels of SFG seropositivity in the Ravenswood area where 10 of the 16 tick samples were qPCR positive for a SFG rickettsia. As only serology was performed it was not possible to determine the species of SFG rickettsia these animals were exposed to. As *I. tasmani* is very common in Tasmania and parasitizes both cats and dogs, 'Candidate R. tasmanensis' is likely to be the causative agent for the seropositivity in some of the cases.

When comparing the gene sequences to those of the validated species mentioned previously¹⁵⁷, 'Candidate R. tasmanensis' did not closely match either of the two validated spotted fever group rickettsiae in Australia (*R. australis* or *R. honei*). Similarly, it was divergent from the two Australian Candidate species ('Candidate R. gravesii', 'Candidate R. antechini'), which are currently being characterized further. In fact, it had the highest phylogenetic similarity to *Rickettsia raoultii* strain Khabarovsk with respect to three of four gene sequences. This particular *Rickettsia* sp. was isolated in the Russian Far

East (more than 10,000 kilometres north of Tasmania) from the tick species *Dermacentor silvarum* and the organism is a known human pathogen in this region¹⁵⁷.

However, the percentage similarities between the two organisms' gene sequences were well below the threshold defined by Fournier et al⁸¹ and based on these results we propose to give this *Rickettsia* sp. a *Candidatus* status and formally name it '*Candidatus* R. tasmanensis' after the geographic location from which it was originally detected.

To validate '*Candidatus* R. tasmanensis' as a novel species, isolation of the agent and subsequent sequencing of the *rrs* gene is required as isolation attempts thus far have been unsuccessful. Multi-gene sequencing of four other qPCR positive *I. tasmani* ticks is also required. This work is currently underway.

As the range of this study was limited to the east side of Tasmania, it would be useful to examine *I. tasmani* ticks from the western half of Tasmania and other parts of Australia for the presence of this rickettsial agent. This would help to determine its true geographical range.

Testing the blood of animals with *I. tasmani* tick parasitism for evidence of SFG rickettsial exposure could also be undertaken and may provide data on the pathogenesis and range of this rickettsia.

Chapter 5. Isolation of *Rickettsia argasii* sp. nov. from the bat tick *Argas dewae*.

This chapter is awaiting ATCC numbers for publication to the *International Journal of Systematic and Evolutionary Microbiology*.

5.1. Abstract

Five isolates of a novel rickettsial species, *Rickettsia argasii* sp. nov. were obtained from the bat tick *Argas dewae* from southern Victoria, Australia. One isolate was characterised by sequencing fragments of five genes. The type strain for this species was designated *Rickettsia argasii* strain T170-B.

5.2. Introduction

In September 2005 a number of *Argas dewae* ticks (a soft tick from the family *Argasidae*¹²⁵), were collected from Organ Pipes National Park (OPNP) in southern Victoria, Australia. The ticks had been found in the roosting boxes of microbats, primarily *Chalinolobus gouldii* and *Vespadelus* spp. Additional ticks were collected from the same sampling point in October and December 2008.

To date three characterised and three 'Candidatus' Spotted Fever Group (SFG) rickettsial species have been identified in Australia; *R. australis* (Queensland Tick Typhus)⁹, *R. honei* (Flinders Island Spotted Fever)²⁴², *R. felis* (cat flea typhus)²²¹, 'Candidatus *R. gravesii*'¹⁷⁵, 'Candidatus *R. antechini*'¹⁷⁴ and 'Candidatus *R. tasmanensis*'¹²⁰. The vectors for five of the six rickettsiae were *Ixodidae* spp. ticks, the exception being *R. felis*, which was detected in the cat flea (*C. felis*).

Worldwide, the presence of SFG rickettsia in soft ticks of the Family *Argasidae* is uncommon with few reports on the presence of SFG rickettsia in *Ornithodoros* spp. and *Carios* spp. ticks^{53, 205}. While there have been reported cases of other vector borne pathogens such as *Coxiella burnetti*¹⁴⁵, *Borrelia* sp.⁵⁶ and *R. bellii*¹⁸⁸ in *Argas* spp. ticks, there are currently no reports of SFG rickettsiae in these ticks.

5.3. Materials and methods

The ticks were processed using methods described in **section 2.1.2** and half of the homogenate was used to isolate and culture the organism using methods described in **section 2.2**. The remaining homogenate was subjected to DNA extraction using the methods described in **section 2.4.1**. Amplification of the *rrs*, *gltA*, *rOmpA*, *rOmpB*, and *sca4* genes was performed on the isolate using methods described in **section 2.4.4**. Amplicons were cloned using the methods described in **section 2.4.5** and sequences were assembled, edited and analysed using methods described in **section 2.4.6**.

5.4. Results

Rickettsial DNA was detected in 70% (7/10) of the *A. dewae* ticks using a *gltA* specific qPCR assay²³⁸. The majority of the ticks collected were female, so no correlation between the sex of the ticks and presence of rickettsia could be determined.

The T170B strain was successfully isolated into VERO cell culture from one of the ticks using the methods described in section 2.2.

Gene sequences amplified from the *A. dewae* isolate were compared to validated sequences mentioned previously¹⁵⁷. The results showed the closest phylogenetic relative for the *gltA*, *rOmpB* and *rOmpA* genes as *R. heilongjiangensis*, with sequence similarities of 99.5% (1093bp/1098bp), 99.1% (4836bp/4881bp) and 98.1% (520bp/530bp) respectively. For the *rrs* and *sca4* genes *R. japonica* was closest with sequence similarities of 99.9% (1420bp/1421bp) and 99.2% (2877bp/2901bp) respectively. The gene sequence analyses using the neighbor-joining algorithm for the five genes are shown below (Figure 5 - Figure 9).

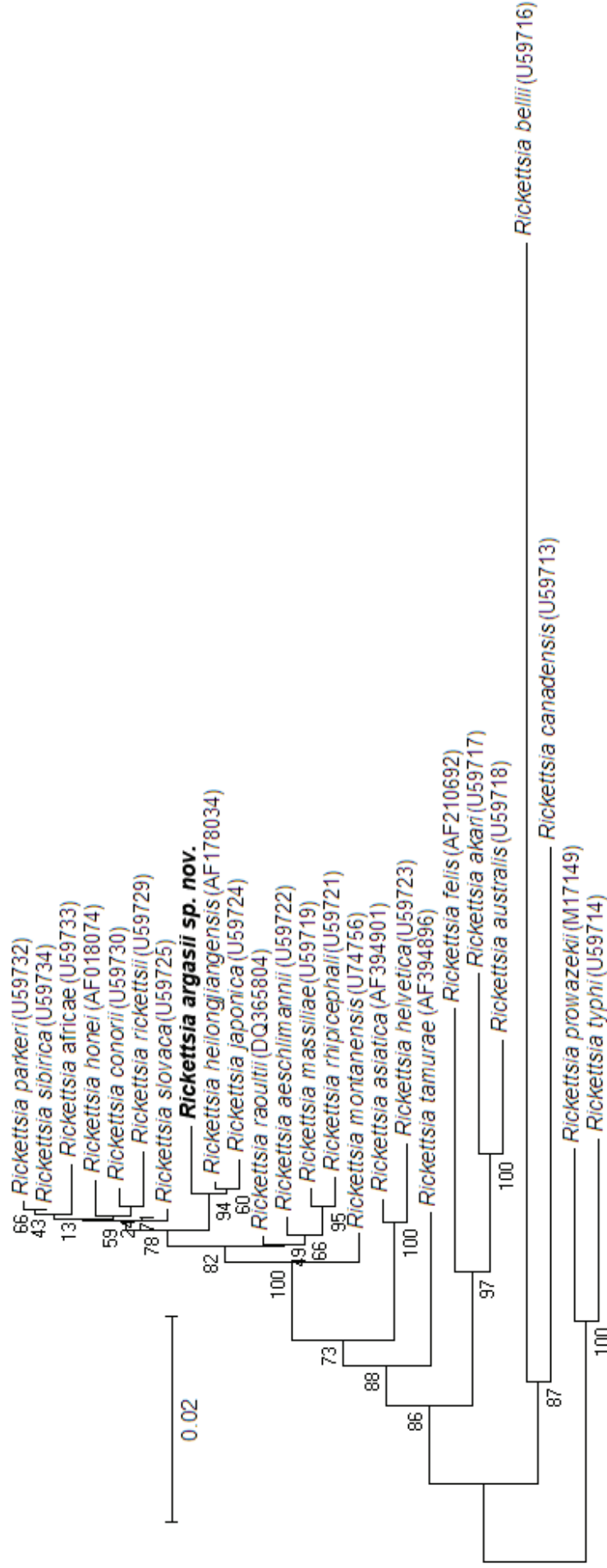


Figure 5. Phylogenetic tree showing the relationship of a 1,098bp fragment of the *gltA* gene of *Rickettsia argasii* sp. nov. to all validated rickettsial species. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 2% nucleotide divergence.

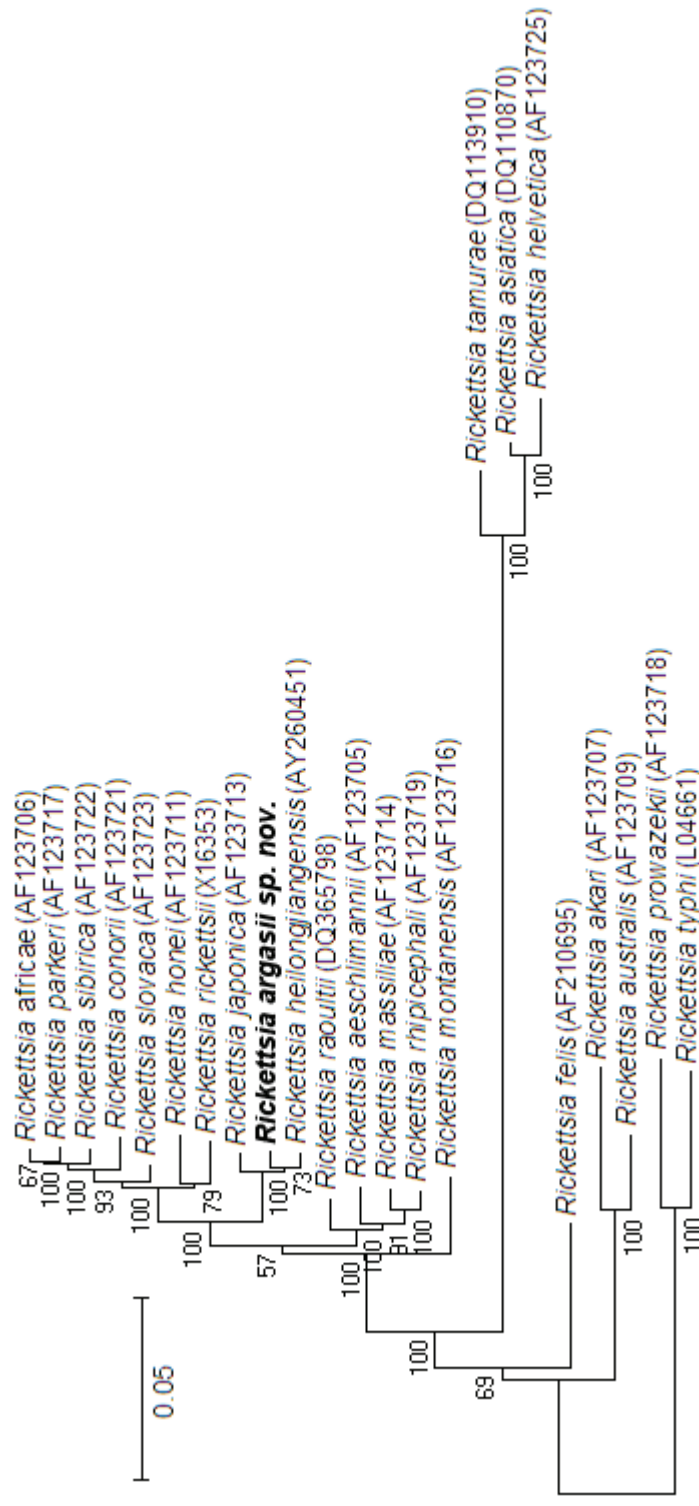


Figure 6. Phylogenetic tree showing the relationship of a 4,881bp fragment of the *rOmpB* gene of *Rickettsia argasii* sp. nov. to all validated rickettsial species. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 5% nucleotide divergence.

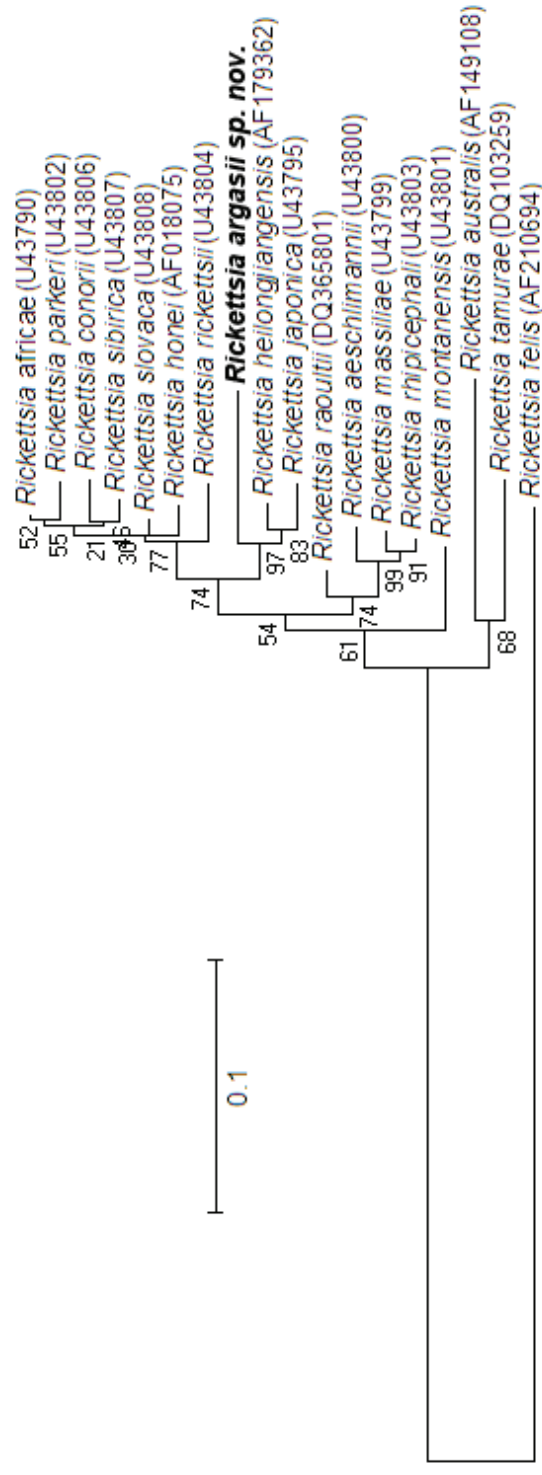


Figure 7. Phylogenetic tree showing the relationship of the *rOmpA* gene of *Rickettsia argasii* sp. nov. to all validated rickettsial species. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 10% nucleotide divergence.

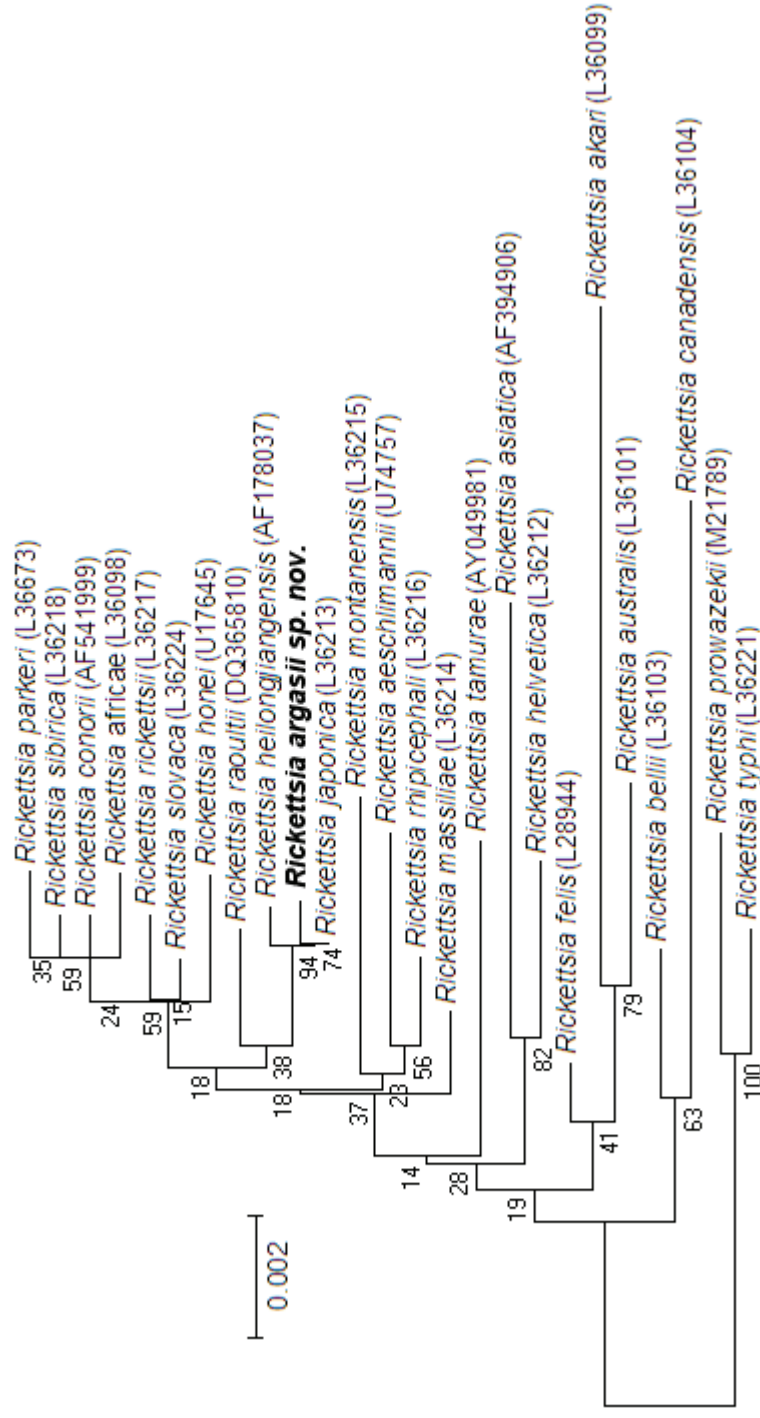


Figure 8. Phylogenetic tree showing the relationship of a 1413bp fragment of the *rrs* gene of *Rickettsia argasii* sp. nov. to all validated rickettsial species. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 0.2% nucleotide divergence.

5.5. Discussion

Of the 10 ticks collected from Organ Pipes National Park, seven (70%) were positive for a SFG rickettsia. Comparison of five genes to those of all validated rickettsial species showed that *Rickettsia argasii* consistently grouped with the *R. japonica* and *R. heilongjiangensis* cluster (Figure 5- Figure 9), with *gltA*, *rOmpB* and *rOmpA* genes of *R. argasii* being most closely related to *R. heilongjiangensis* and the *rrs* and *sca4* genes being most closely related to *R. japonica*. The criteria defined by Fournier *et. al.* (2003) states that for a rickettsial agent to be considered a new species it must not have more than one gene that exhibits a degree of “nucleotide similarity with the most homologous validated species” of equal to or greater than 99.9%, 99.8%, 99.3%, 99.2% and 98.8% for the *gltA*, *rrs*, *sca4*, *rOmpB* and *rOmpA* genes⁸¹. *Rickettsia argasii* exhibited percentage similarities of 99.9%, 99.5%, 99.2%, 99.1% and 98.1% for the *rrs*, *gltA*, *sca4*, *rOmpB*, and *rOmpA*, so by these criteria, only the *rrs* gene fell within the species threshold for our isolate, and therefore satisfies the requirements for classification as a new species.

Furthermore, our analysis of the sequences from the 2003 article on *R. heilongjiangensis*⁸¹ discovered an apparent error in the percentage homology calculations for the *rrs* gene. The correct percentage similarity between *R. japonica* and *R. heilongjiangensis* appears to be 99.9% rather than 98.0% as published. This error changes the outcome of the study as the defined parameters now place the isolate *R. heilongjiangensis* as a strain of *R. japonica*

rather than a new species. Both the *sca4* and *rrs* genes fall within the species threshold. Therefore with this correction in mind, all 5 genes of *R. argasii* align most closely with *R. japonica*, the agent of Oriental spotted fever.

This isolate had the highest phylogenetic similarity to *R. japonica*, which is a known human pathogen²⁵⁶. This combined with the knowledge that some *Argas* spp. ticks are known to aggressively feed on humans⁷⁴ suggests that this new rickettsia may be a potential human pathogen.

Based on the phylogenetic results, we propose to nominate this rickettsial isolate as a novel species and name it *Rickettsia argasii* sp. nov. after the tick genus (*Argas* spp.) from which the isolation was made.

As the range of this study was limited to a single location in central Victoria, it would be useful to examine *A. dewae* ticks from roosting boxes in other locations in Victoria, and throughout Australia, for the presence of this rickettsial agent. This would help to determine the true geographical range of this species.

The likelihood of this agent being present elsewhere is high as bats are known to travel great distances and therefore can potentially spread ticks (with rickettsiae) over great distances.

Testing the blood of bats inhabiting the nesting boxes where the *A. dewae* ticks were found for evidence of SFG rickettsial exposure could also be undertaken and may provide data on whether the bats produce an antibody response to these rickettsiae.

Chapter 6. First cases of probable *Rickettsia felis* (cat flea typhus) in Australia

This chapter has been submitted for publication to the *Medical Journal of Australia* for publication. Changes have been made to the formatting of this chapter to integrate it into the thesis.

6.1. Abstract

This study reports five cases of a rickettsial disease (probably *Rickettsia felis*) in Victoria, Australia. All patients (four from one family) contracted the disease after exposure to fleas from cats. Molecular testing of fleas from cats of the same cohort demonstrated the presence of *R. felis* (but not *R. typhi*). This rickettsial agent has now been confirmed in cat fleas from Victoria and Western Australia.

6.2. Introduction

In 1990, Adams described by a “rickettsia-like microorganism” by electron microscopy in the midgut cells of adult cat fleas (*Ctenocephalides felis*) obtained from a commercial cat flea colony. He noted that these organisms had a well defined cell membrane that was consistent with a rickettsia and were 0.25 to 0.45 μm in diameter and up to 1.5 μm in length. These organisms were

named the “ELB agent” after the laboratory from which the fleas were obtained (EI Labs, Soquel, CA, USA)².

In 1994, the agent was detected in the blood of a patient from Texas who was initially diagnosed with murine typhus (*R. typhi*) using serological methods. With the use of molecular techniques the causative organism was confirmed as *the* ELB agent. This article also suggested that a number of retrospective samples diagnosed as *R. typhi* may in fact have been this agent, as the serological techniques used could not differentiate between these agents²²². Subsequently, human cases have been reported in Europe (France²⁰², Germany²⁰⁸ and Spain^{173, 184}), South and Central America (Mexico²⁸² and Brazil²⁰²), Africa (Tunisia²⁸⁵) and Asia (Thailand¹⁸⁰, South Korea⁴⁴, Laos¹⁸⁹ and Taiwan²⁵³).

In 1996, the name *R. felis* was proposed¹⁰⁷, although it was not formally named, as no strain had been deposited in any official culture collection. Isolation of the agent was reported on several occasions^{107, 198, 199}, however contamination with *R. typhi* hampered initial characterization of the organism^{135, 202}. In 2001, complete characterization and isolation of a pure culture of *R. felis* was achieved²⁰².

Rickettsia felis reacts serologically as a typhus group rickettsia and was therefore placed in this group until 2001, when it was found to contain an OmpA gene. After this discovery it was subsequently reclassified as a member of the

spotted fever group rickettsiae²⁷. However it has recently been reclassified as a member of the transitional group rickettsiae⁹¹.

The primary vector and reservoir for *R. felis* is the cat flea (*C. felis*)²⁴¹, although it has been found in various other flea species including *Xenopsylla cheopis*^{73, 121}, *Ctenocephalides canis*^{89, 241}, *Archaeopsylla erinacei*^{21, 89, 90}, *Anomiopsyllus nudata*²⁴¹, *P. irritans*²⁴¹, *Tunga penetrans*²¹⁹, *Echidnophaga gallinacea*²¹⁹ and *X. brasiliensis*²¹⁹, as well as trombiculid mites⁴⁵. A article from 2003 reported the presence of *R. felis* in several tick species although this is the only report of *R. felis* in ticks¹¹⁸.

Since its discovery in 1990, *R. felis* has been detected in vectors worldwide¹⁸⁵ including; America^{2, 171}, Europe¹⁴⁷, Africa²⁰², Asia¹⁸² and Australasia^{132, 221}.

Currently, only a single Australian report of *R. felis* has been published, from *C. felis* fleas collected in Western Australia²²¹. This study reports the first detection of *R. felis* in Victoria and the first probable cases of human infection by *R. felis* in Australia.

6.3. Case Study

Patient B, a previously well 9-year-old girl, was admitted to the Royal Children's Hospital, Melbourne, Australia, with severe abdominal pain, fevers to 39°C and a non-pruritic erythematous macular rash, initially present on the trunk and then spreading to the upper limbs and face (figure 1). The patient described a prodrome of five days of fever, malaise, with occasional vomiting and diarrhoea.

She was appropriately immunised, with no drug allergies or regular medications.

Initial examination revealed an unwell-looking girl with pitting oedema of the ankles and a generalised macular rash. There was no hepato-splenomegaly or significant lymphadenopathy. Initial laboratory tests demonstrated leukopenia (WBC=3.0 x 10⁹/L NR 4.5-13.5), lymphopenia (lymphocytes 0.42 x 10⁹/L NR 1.5-6.5), thrombocytopenia (platelets= 38 x 10⁹/L, NR 150-400), hyponatremia (Na+=133 mmol/L, NR 135-145), elevated transaminases (AST=168 IU/L, ALT=177 IU/L, NR <55) and hypoalbuminemia (19g/L, NR 33-47). The patient was commenced on ticarcillin/clavulanic acid and gentamicin. Urine and blood cultures were sent to the laboratory, as well as serology for a range of infectious diseases.

The patient lived with her parents and two siblings in suburban Melbourne, Victoria, on a hobby farm next to a wooded reserve notable for stagnant water and mosquitoes. The family had many pets, including a dog, goat, ducks, budgerigars, mice and a domesticated rat. They had never travelled outside the Australia, nor had recent overseas visitors. Three weeks prior to the illness, the family acquired a pair of kittens (cat #1 and #2) from a farm at Lara, Victoria, one of which was given to a neighbour (patient A).

Patient B continued to be persistently febrile with severe abdominal pain. She remained thrombocytopenic, with worsening hepatic function, coagulopathy, hyponatremia and hypoalbuminaemia. On day 3 of her admission, she developed pulmonary oedema, requiring a short ICU stay during which she

received azithromycin, albumin, and frusemide as well as intensive supportive therapy. She was given intravenous immunoglobulin (IVIG) 2g/kg for possible Kawasaki disease.

On day 3 of Patient B's hospitalisation, her 8-year-old sister (Patient C) presented with fevers to 40°C, mild abdominal pain and a rash on her torso. Examination revealed a well girl with florid facial flushing; a macular rash spreading to the limbs; tender cervical lymph nodes, and a mildly tender abdomen.

Patient C's initial laboratory tests were notable only for leukopenia of 4.5 and hyponatremia of 132. She was commenced on ticarcillin/clavulanic acid and gentamicin. Patient C became thrombocytopenic over 48 hours, with worsening abdominal pain, hyponatremia and elevated transaminases. She was given IVIG 2g/kg for possible Kawasaki disease and improved rapidly.

On day 8, Patient D, the girls' 4-year-old brother, presented with a fever of 39.6°C and five erythematous macules on his legs and trunk. He was otherwise well.

Laboratory tests for Patient D showed a leukopenia of 4.0, with no other abnormalities. He was admitted for observation without treatment.

The three siblings were discharged home on day 11 of Patient B's hospitalisation, with no definitive diagnosis. Patients C and D continued to spike fevers for one week, but remained otherwise well.

A phone review on day 18 revealed that all children were well and afebrile. However, their maternal grandmother (Patient E) had had 3 days of fever and

rigors and had been admitted to another hospital for observation. Upon advice from the sibling's doctor, she was administered doxycycline and subsequently improved. A neighbour (Patient A) had also been unwell 2 days prior to patient B with a non-specific febrile illness, which had settled by the time of the sibling's investigation. She was the initial case in the cluster.

Both the family and the neighbour had adopted one of the two kittens recently acquired from a farm in Lara. Both cats had flea infestations. All patients had had extensive close contact either or both cats.

Additional serological tests were performed on all patients and the parents of Patients B, C and D along with the now de-fleaed surviving cat #1 (cat #2 was euthanased before blood could be taken).

6.4. Methods

6.4.1. Serology

Serological testing was performed on the seven human and one feline sera using methods described in **section 2.5.1**.

6.4.2. PCR

DNA was extracted from the buffy coat of one of the patients and on pools of crushed cat fleas (*C. felis*) collected from cats from the same farm as the patient's cat (which was euthanased prior to this study) as described in **section 2.4.1** and a real-time PCR was performed on the extracted DNA samples²³⁸.

A fragment of the citrate synthase gene was amplified as described previously²¹⁸, then cloned and sequenced using methods described in **section 2.4**.

6.4.3. Molecular Characterisation

The sequencing results were analysed using methods described in **section 2.4.6**.

6.4.4. Attempted Isolation and Culture

A method modified from Raoult *et. al.* (2001) was used in an attempt to isolate the organism²⁰². *Ctenocephalides felis* fleas were pooled and surface sterilised with a 5 minute immersion in 70% ethanol, followed by 3 rinses in sterile PBS. They were then aseptically divided into groups of 5 fleas and placed in a 1.5ml micro-centrifuge tube containing 400µl of sterile PBS and homogenised using a disposable micro-centrifuge pestle (Edwards Instruments Co., Australia). The flea homogenate was aliquoted into 24 well trays containing VERO (green monkey epithelial cell), DH82 (dog macrophage) and XTC-2 (south African clawed frog epithelial cell) cell lines in RPMI media (GIBCO, USA) containing 4µg/ml trimethoprim and 20µg/ml sulfamethoxazole (GlaxoSmithKline, United Kingdom) and centrifuged for 30 minutes at 40g. The trays were then incubated at 28°C for 1 month with weekly changes of media. Some flea homogenates were passed through a 0.45µm or 0.22µm filter and inoculated into antibiotic-free cell cultures.

Animal inoculation was also attempted on the *C. felis* flea samples. The flea homogenate was inoculated intraperitoneally into SCID mice, and the mice were observed daily for 6 weeks. At the conclusion of this time they were euthanased and their spleens were aseptically removed for rickettsial PCR analysis.

6.5. Results

6.5.1. Serology

Initial serological analysis undertaken on the two female siblings showed the presence of typhus group antibodies. A month later serological testing was repeated for patients B and C as well as their younger male sibling (patient D), their grandmother (patient E), a neighbour (Patient A) who became unwell after exposure to the kittens and the children's mother and father. The three children, grandmother and neighbour showed high levels of typhus group antibodies, and patient C showed clear evidence of sero-conversion (Table 6), while both parents were negative for rickettsial antibodies. Serology undertaken on cat #1 also showed the presence of typhus group rickettsial antibodies (Table 6).

Table 6. Serology results from five patients and a cat post-exposure to a rickettsial agent.

Patient	Sex	Age	Sampling Date			
			April-09	May-09	June-09	July-09
A	F	63	ND	<i>R. p.</i> : 1/16384 <i>R. t.</i> : 1/16384	ND	ND
B	F	9	<i>R. p.</i> : 1/1024 <i>R. t.</i> : 1/2048	<i>R. p.</i> : 1/8192 <i>R. t.</i> : 1/8192	ND	ND
C	F	8	<i>R. p.</i> : 1/128 <i>R. t.</i> : <1/128	<i>R. p.</i> : 1/16384 <i>R. t.</i> : 1/16384	ND	ND
D	M	4	ND	<i>R. p.</i> : 1/16384 <i>R. t.</i> : 1/16384	ND	ND
E	F	59	ND	<i>R. p.</i> : 1/1024 <i>R. t.</i> : 1/1024	<i>R. p.</i> : 1/2048 <i>R. t.</i> : 1/2048	ND
Cat #1	F	<1	ND	ND	ND	<i>R. p.</i> : 1/512 <i>R. t.</i> : 1/512

ND = not done *R. p.* = *R. prowazekii* *R. t.* = *R. typhi*

6.5.2. Molecular Analysis

DNA was extracted from the serum of patient C (buffy coat was not available) and on pools of crushed cat fleas (*Ctenocephalides felis*) collected from cats from the same cat cohort in Lara, Victoria. By this stage of the investigation one cat (#2) was dead and the other (#1) no longer had fleas. A rickettsial real-time PCR was performed on the extracted DNA samples as previously described²³⁸, with the fleas (but not the patient) testing positive for rickettsial DNA.

The citrate synthase gene sequence was compared to validated rickettsial species⁸¹. The sequence analysis showed the closest phylogenetic similarity with *Rickettsia felis*, with a sequence similarity of 99.7% (1074/1077). The citrate synthase gene sequence analysis using the neighbor-joining algorithm is shown in Figure 10.

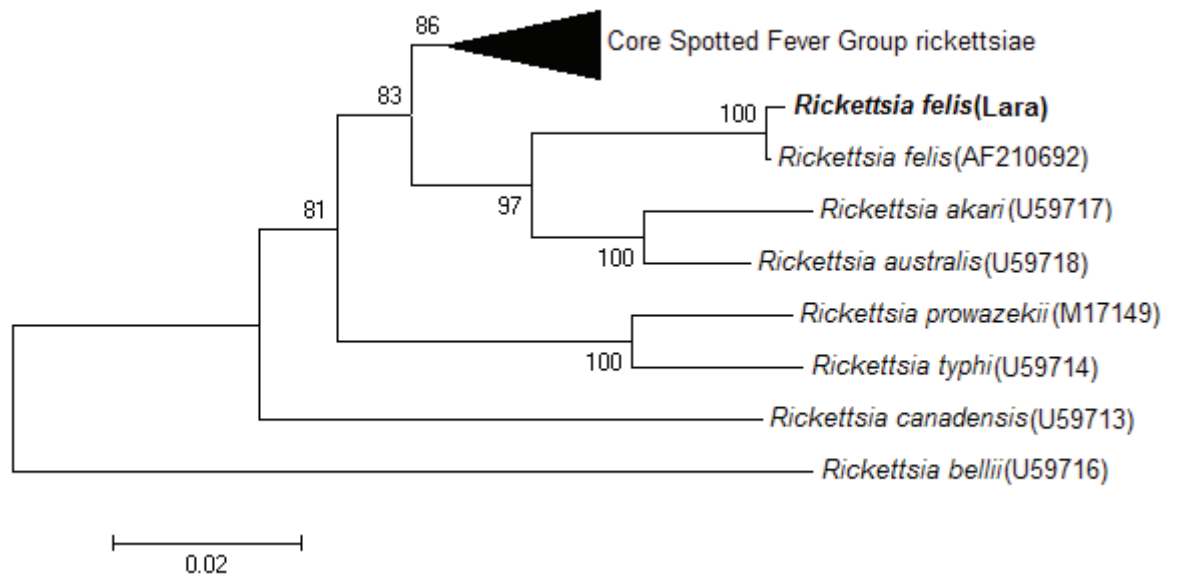


Figure 10. A condensed phylogenetic tree showing the relationship of a 1077bp fragment of the *gltA* gene of *Rickettsia felis* (Lara) among other validated rickettsial species, with the core spotted fever group rickettsiae truncated. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 2% nucleotide divergence.

6.5.3. Attempted Isolation and Culture

After a one month incubation of the inoculated cell lines, IF and qPCR analysis revealed negative results for all cultures. Analysis of the spleens of the inoculated SCID mice using qPCR also revealed negative results for all samples. Thus the strain was not isolated.

6.6. Discussion

The five cases described are the first reported cases of probable human *R. felis* infection in Australia. *R. felis* has been previously detected molecularly in cat and dog fleas from Western Australia (10). These cases are the first molecular evidence of *R. felis* in cat fleas in Victoria. There are reported human cases due to *R. felis* from most parts of the world (6, 7, 9, 11).

Whilst genetically a member of the spotted fever rickettsia group, *R. felis* behaves clinically and serologically like a typhus group rickettsiae and is flea-transmitted. Antibodies induced by *R. felis* react with typhus group rickettsiae in serological tests, rather than with spotted-fever group rickettsiae. A petechial rash is infrequent and a macular or maculopapular rash is present in only 50% of patients (Figure 1). The high attack rate and noted severity of infection noted in this cluster may well be due to the heavy flea infestation that was reported. The observed response to IVIG has not been reported elsewhere and only 2 patients (B and C) received antimicrobial therapy with known activity against *Rickettsia*. Resolution without therapy is well described in rickettsial infection. The five patients showed a strong positive result for the presence of typhus group antibodies, with patient C showing a clear seroconversion, which was consistent with either recent acute *R. felis* or *R. typhi* infections²²². Cat #1 was also positive for typhus group rickettsia antibodies, and while exposure to either *R. felis* or *R. typhi* may cause this response in the cat fleas only *R. felis* DNA was detected. Analysis of cat blood for rickettsial DNA was negative, which is common with *R. felis* infection in cats⁷³. A previous experimental exposure of

cats to *R. felis* positive fleas resulted in 13 of the 16 cats testing positive by serology (IF) but only 5 of the 16 positive by PCR ²⁷⁵. The cat still had antibodies to *R. felis* but had either cleared the infection or the organism was present in tissues other than peripheral blood.

Both cats were infested with fleas when the family and neighbour obtained them but one had been euthanased and the other treated topically with insecticide by the time its blood was taken. *C. felis* fleas taken from other cats of the same group in Lara, Victoria, including its nuclear family contained DNA which was identified as *R. felis* by PCR amplification and sequencing (Figure 2). *R. typhi* DNA was not detected in the cat fleas.

The human cases reported in this study were only identified serologically, and as the clinical presentations of both *R. typhi* and *R. felis* are similar, *R. typhi* cannot be completely ruled out as the causative agent. However, given the molecular data from the cat fleas, *R. felis* is the more likely diagnosis.

This appears to be the first reported case of human infection with *R. felis* in Australia. The incidence of this infection may have been underestimated in the past due to misdiagnosis as *R. typhi*. As human infection with *R. felis* was previously unknown in Australia, any previous cases of infection with raised typhus group antibodies were probably reported as *R. typhi*, when the causative agent may well have been *R. felis*. This confusion has been seen in other studies ^{21, 73}.

Chapter 7. Isolation of a highly variant *Orientia* species (*O. chuto* sp. nov.) from a patient returning from Dubai

This manuscript is in progress and will be submitted for publication to the *Journal of Clinical Microbiology*. Changes have been made to the formatting of this chapter to integrate it into the thesis.

7.1. Abstract

In July 2006, an Australian tourist returning from Dubai in the United Arab Emirates developed acute scrub typhus infection. Her symptoms included; fever, myalgia, headache, rash and an eschar. *Orientia tsutsugamushi* serology demonstrated a 4-fold rise in antibody titres in paired sera (1:512 to 1:8192) with the Gilliam strain reacting the strongest. An isolate was obtained. Comparisons of the 16S rDNA (*rrs*), and 47kDa with other *O. tsutsugamushi* strains demonstrated significant genetic differences. The closest homology with the 16S RNA sequence of *O. chuto* was with three strain of *O. tsutsugamushi* (Ikeda, Kato and Karp) with a nucleotide sequence similarity of only 98.5%. The closest homology with the 47kDa sequence was with *O. tsutsugamushi* strain Gm47 with a nucleotide similarity of 82.3%. However the closest validated strain was Ikeda with a nucleotide similarity of 82.2% for the 47kDa gene.

This new isolate appears to be the most divergent strain of *Orientia* sp. reported to date. Because of this and its geographically unique origin we believe that it should be considered to be a new species of the genus *Orientia*. Therefore we have named this new isolate *Orientia chuto*, with 'chuto' being the Japanese word for 'Middle East', and the prototype strain of this species is strain Churchill named after the patient whom it was isolated.

7.2. Introduction

Scrub typhus, caused by the bacterium *Orientia tsutsugamushi* is transmitted by chigger mites (primarily *Leptotrombidium* spp.). It is known to occur within an 8 million plus square kilometre region, from Siberia in the north, the Kamchatka Peninsula in the east, to Afghanistan in the west down to Australia in the south (Queensland, the Northern Territory and Western Australia and parts of Oceania^{50, 94, 271}). The *Orientia* spp. that this study focuses on originated in Dubai, United Arab Emirates, which is over 500 kilometres west of the known scrub typhus region (Figure 11). High levels of scrub typhus infection occur primarily in the tropics, usually in areas of dense scrub, although the organism has also been found in more temperate zones and even semi arid climates²⁵². It is estimated that over one million cases of scrub typhus occur each year²⁷², although accurate incidence rates are hard to obtain as infection often occurs in rural areas where the facilities and skills required to diagnose it are unavailable. In some endemic regions studies has shown that over 80 percent of the adult population had antibodies to *O. tsutsugamushi*³⁵.

In 1995, after extensive re-analysis of the genus *Rickettsia*, the organism *Rickettsia tsutsugamushi* was re-classified under the new genus *Orientia* and was renamed *Orientia tsutsugamushi* gen. nov.²⁴⁴, this is the only species, with numerous strains. In the past, new strains of *O. tsutsugamushi* were compared to only Kato, Karp and Gilliam serotypes⁷⁰. With advances in diagnostic methods, the number of different serotypes has increased dramatically¹²⁹. However, all of the previous strains were genetically very similar¹⁶⁹.

This study focuses on a divergent isolate of *Orientia*. It was grown *in vitro* and analysed to determine its degree of genetic divergence. A novel qPCR was developed to also enable detection of this new isolate.

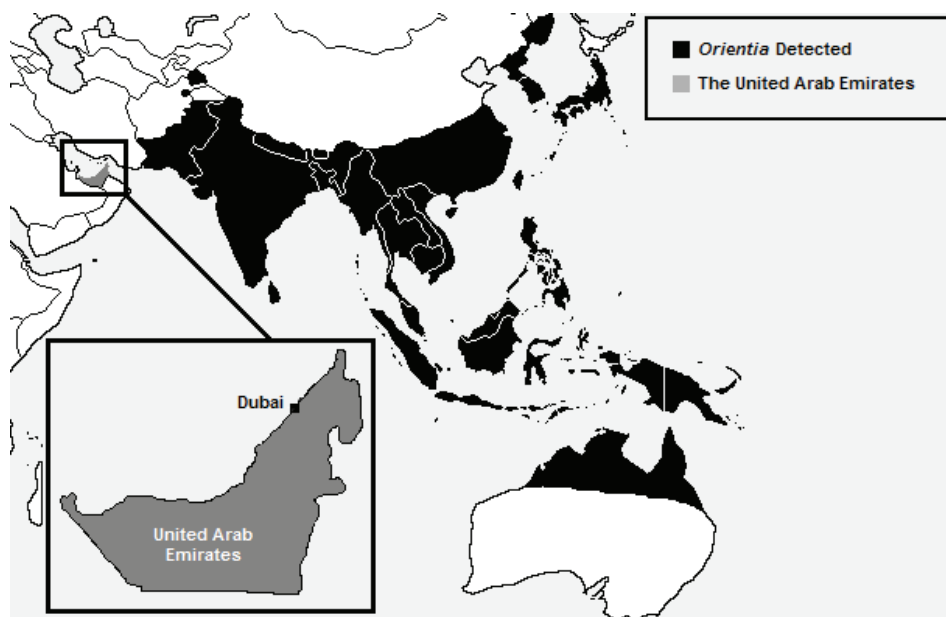


Figure 11. A regional map showing the distribution of scrub typhus and the location of Dubai within the United Arab Emirates.

7.3. Clinical case history

A 52-year-old female presented to her local general practitioner (GP) in mid July 2006. She had travelled to Dubai and the United Kingdom prior to the onset of her illness. During her stay in Dubai she visited a stable where there were horses, dogs and cats (day 0). She noticed an eschar on her abdomen 11 days later (Figure 12). She developed lymphadenopathy by day 16 and complained of general myalgia (day 17) followed by fever and rash (day 18). This incubation period is slightly longer than what is typically seen with scrub typhus group infections (8 to 10 days)²³¹. On day 21 she developed a maculopapular rash over her abdomen and complained of headache, pain behind her eyes, generalised myalgia and backache. Her full blood examination (taken on day 24) showed elevated levels of liver function tests and a slight rise in her C-reactive protein (CRP) but was otherwise unremarkable. Her GP misdiagnosed her with a viral illness.



Figure 12. Eschar on the abdomen of the patient.

She was seen by an infectious diseases physician on day 24, who found that she was afebrile with faint macules on her abdomen and an eschar on her upper abdomen. She had mild hepatomegaly but no splenomegaly. Blood tests for a number of pathogens were performed and showed a positive antibody titre of 1/512 for STG rickettsia (Figure 13). A strain of what was assumed to be *Orientia tsutsugamushi* was later isolated from a blood sample taken on day 24. The physician diagnosed her with a rickettsial infection and started doxycycline treatment (100mg twice daily) for 14 days. Two days after treatment commenced (day 26) the patient was admitted to hospital due to ongoing fevers and lethargy. A full blood examination on admission showed an elevation in her overall white cell count with an increase in her monocyte and lymphocyte count. A blood film also showed the presence of numerous atypical (reactive) lymphocytes. The erythrocyte sedimentation rate (ESR) was normal but the

CRP was elevated. Liver function tests revealed a rise in her alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (γ GT). After completion of the 14 day course of doxycycline her liver function had normalised. By day 34 her fever and sweats had gone and her myalgia and rash were noticeably reduced. By day 43 she reported feeling “markedly better”, although still quite lethargic. Her STG rickettsia serology titre peaked at 1/8,192. Serology taken on day 57 showed a decrease in titre to 1/1024 (Figure 13). By day 90 the patient was still reporting continued lethargy.

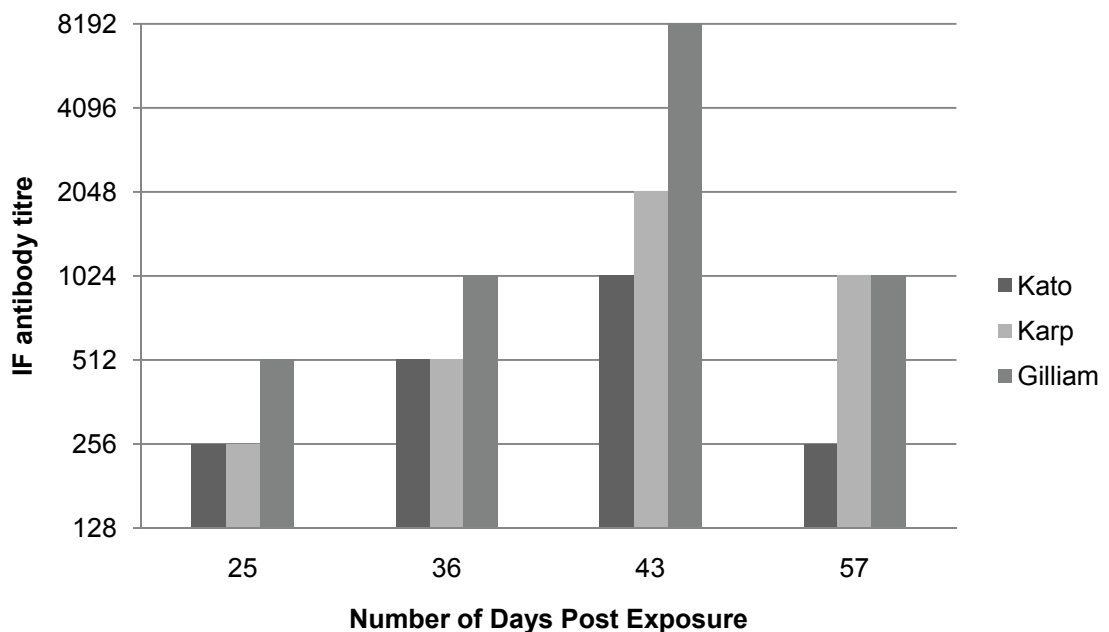


Figure 13. Scrub Typhus serology, showing a marked change in antibody titres to three strains of *O. tsutsugamushi* over a 57 day period.

7.4. Materials and methods

7.4.1. Microimmunofluorescence assay (IFA)

Serum taken from the patient was tested for the presence of STG antibodies using the IFA described in **section 2.5.1**.

7.4.2. Culture

An EDTA blood tube was collected (along with the first serum sample) prior to doxycycline treatment. Successful Isolation and culture of the organism was performed using methods described in **section 2.2**.

7.4.3. DNA extraction and PCR assays

DNA was extracted using methods described in **section 2.4.1**.

7.4.3.1. 16S rRNA gene

A 1354bp fragment of the 16S rRNA gene was amplified using a previously described assay⁸¹.

7.4.3.2. 47kDa gene

Primers were designed by Allen Richards from the Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, USA (unpublished) to amplify a 1404bp fragment of the 47kDa gene. This involved aligning the 47kDa sequences of 21 strains of *O. tsutsugamushi* to identify conserved regions within the gene that would be suitable as primer targeting

regions. The specific primers that targeted the 47kDa gene of *O. chuto* strain Churchill (Chur 627F and Chur 669F) were chosen after a partial sequence was obtained (Table 7).

A 25µl total volume reaction mixture was prepared containing 1.5 µl of DNA template, PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) and 0.3 µM of forward and reverse primers. One microliter of PCR products were used as templates in the nested PCR reactions. Amplification was performed by initially heating to 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 51°C (or 54°C for the nested PCR) for 30 sec and 68°C for 1 min, followed by a final 7 min at 72°C. The PCR products were electrophoresed through a 1% agarose gel, stained with ethidium bromide and the bands were viewed using a UV illuminator.

Table 7. Primer sequences used to amplify the 47 kDa genes (Richards et al.).

Primer Name	Gene	Sequence (5' – 3')
Ot-263F	47 kDa	GTG CTA AGA AAR GAT GAT ACT TC
Ot-1133R	47 kDa	ACA TTT AAC ATA CCA CGA CGA AT
Chur 627F	47 kDa	GCG GGA TAT AGG TAG TTC AA
Chur 669F	47 kDa	TAT TCA AAC TAA TGC TGT TGT GC
Otr471404R	47 kDa	GAT TTA CTT ATT AAT RTT AGG TAA AGC AAT GT

7.4.4. Sequencing

Amplicons were cloned using the methods described in **section 2.4.5** and sequences were assembled, edited and analysed using methods described in **section 2.4.6**.

7.4.5. qPCR design

A qPCR targeting the *rrs* gene of *O. chuto* strain Churchill (Table 8) was designed and validated using methods described in **section 2.4.2**.

Table 8. qPCR primer and probe set sequences targeting the 16S rRNA gene.

Name	Sequence (5' – 3')
CH-F	GGA GGA AAG ATT TAT CGC TGA TGG
CH-R	TAG GAG TCT GGG CCG TAT CTC
CH-P	FAM d(TGT GGC TGT CCG TCC TCT CAG ACC) BHQ-1

7.5. Results

7.5.1. Serology

Serum samples were collected from the patient on days 25, 26, 43 and 57. On day 25 the serological results showed a titre of 1/512 against the Gilliam serotype and 1/256 against the Kato and Karp serotypes. The levels peaked by day 43 with antibodies towards the Gilliam serotype showing a 5 fold doubling increase in titres (1/8192). Antibody levels towards both Kato and Karp serotypes showed a 2 fold (1/1024) and 3 fold (1/2048) doubling dilution increase in titres respectively. By day 57 the levels had dropped to 1/256 for Kato and 1/1024 for both Karp and Gilliam serotypes respectively (Figure 13).

7.5.2. Culture

The Vero cell culture inoculated with pre-antibiotic treated buffy coat from the patient (day 25) was incubated for two weeks. After this time it was tested by direct IF, showing growth of *Orientia* spp. within the Vero cells.

7.5.3. Molecular analysis

Pairwise analysis of this isolate with various strains of *O. tsutsugamushi* showed that *O. chuto* had the highest level of divergence in both the 16S rRNA (rrs) and 47kDa genes (Table 9). The analysis of the 16S rRNA sequence showed the closest phylogenetic relative as *O. tsutsugamushi* strains Ikeda, Kato and Karp with sequence similarities of 98.5%. The analysis of the 47kDa gene compared to all sequences that were available on the NCBI database showed the closest phylogenetic relative to be *O. tsutsugamushi* strain Gm47 with a percentage similarity of 82.3% although the closest validated relative was *O. tsutsugamushi* strain Ikeda with a similarity of 82.2% (Table 9).

Table 9. Percentage pairwise divergence plot of *O. chuto* strain Churchill with various strains of *O. tsutsugamushi* showing the significant level of divergence of *O. chuto* strain Churchill.

16S rRNA	1	2	3	4	5	6	7	8	9	10
1-Churchill										
2-Ikeda	1.5									
3-Boryong	1.6	0.3								
4-Kato	1.5	0.0	0.3							
5-Karp	1.5	0.2	0.1	0.2						
6-Gilliam	1.7	0.5	0.2	0.5	0.3					
7-Kuroki	1.6	0.3	0.0	0.3	0.1	0.2				
8-Kawasaki	1.9	0.4	0.5	0.4	0.4	0.6	0.5			
9-Shimokoshi	2.0	1.0	1.1	1.0	1.0	1.1	1.1	0.9		
10-Litchfield	1.9	0.6	0.4	0.6	0.4	0.5	0.4	0.6	1.1	

47kDa	1	2	3	4	5	6	7
1- Churchill							
2-Gm47	17.7						
3-Ikeda	17.8	0.6					
4-Br47	17.9	3.3	3.1				
5-Kp47	18.0	2.3	2.0	3.2			
6-Pkt5	18.0	0.8	0.1	3.2	2.1		
7-Boryong	18.2	3.1	2.8	0.8	3.2	3.0	

The phylogenetic relationships between each of the gene sequences of *O. chuto* strain Churchill and various strains of *O. tsutsugamushi* were analysed using the algorithms mentioned above. The 16S rRNA and 47kDa gene sequence analyses using the neighbour-joining algorithm are shown in Figure 14.

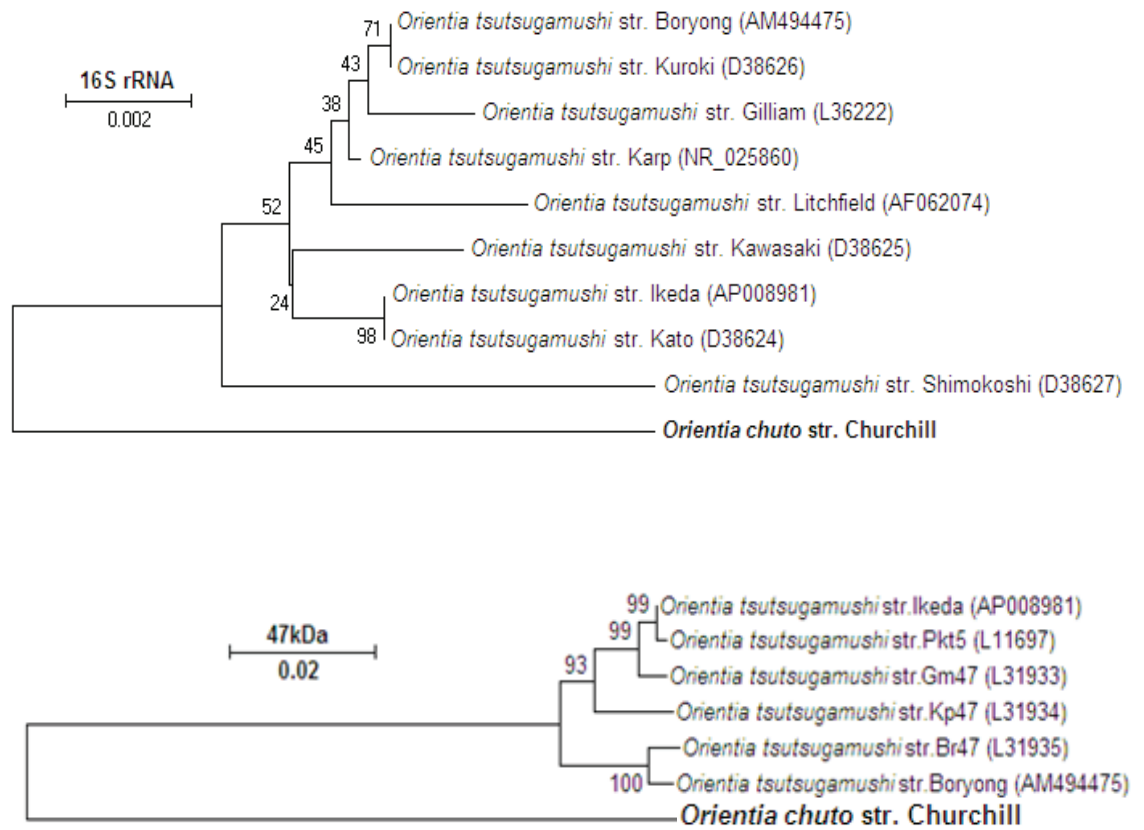


Figure 14. Phylogenetic trees showing the relationship between the 16S rRNA and 47kDa genes of *Orientia chuto* strain Churchill to various *O. tsutsugamushi* strains. The tree was prepared using the neighbor-joining algorithm, within the Mega 4 software. Bootstrap values are indicated at each node. The scale bars represents a 0.2% and 2.0% nucleotide divergence for the 16S rRNA and 47kDa gene respectively.

7.5.4. qPCR

A real-time PCR assay (targeting the 16s rRNA gene) performed on the *O. chuto* culture was positive and a 141 base pair amplicon was observed when the product was run on an agarose gel. The sensitivity of the assay, determined by performing real-time PCR on titrated plasmid samples ranging from 9.9×10^{10} to 9.9 copies, was shown to be 9.9 template copies per reaction. The C_t values ranged from 3.52 (9.9×10^{10} copies) to 35.86 (9.9×10^0 copies) (Figure 15).

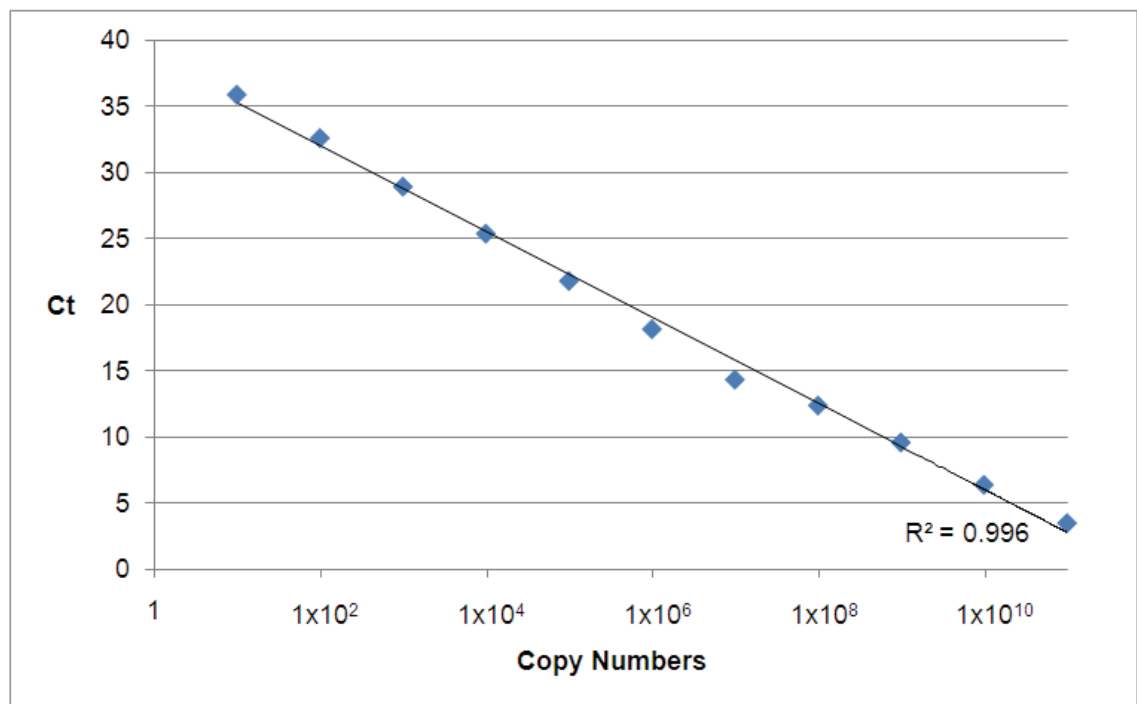


Figure 15. A standard curve showing the C_t versus the number of copies of the template containing plasmid.

When tested with *O. tsutsugamushi* strains Kato, Karp and Gilliam, the same assay produced a positive result for all 3 strains and a band around the 141 base pair mark was observed when the products were run on an agarose gel. The assay produced negative results when tested against the DNA of other medically important bacteria²³⁸.

7.6. Discussion

In this study, the patient contracted scrub typhus in Dubai within the United Arab Emirates, which is well outside the recognised “tsutsugamushi triangle”. Typically scrub typhus is considered a tropical disease with the majority of cases occurring in rural Asian tropics, although cases have occasionally been reported in temperate zones²⁷¹. The habitat within which transmission usually occurs are areas where scrubland has been allowed to grow after deforestation²⁷¹. The natural environment of the United Arab Emirates is desert with scant vegetation. More recently, however, the environment has been altered considerably with millions of trees having been planted. Even so, this would not be considered an ideal environment for mites and *O. tsutsugamushi*.

Samples collected from the patient were positive for the presence of scrub typhus group antibodies using IFA serology, showing a clear rise in titre. Testing of the patient’s acute blood sample for the presence of *O. tsutsugamushi* DNA was positive using a new qPCR assay that was designed from the 16S rRNA gene sequence. The Vero culture inoculated with the same pre-antibiotic buffy-coat sample used for the qPCR assay was examined after two weeks of growth

using direct IF and revealed the presence of a scrub typhus group rickettsiae growing within the cytoplasm of the VERO cells. qPCR analysis of the culture using the new assay was also positive. This new qPCR assay has the potential to be used in a clinical setting for the diagnosis of both *O. chuto* and *O. tsutsugamushi* as it detected all strains tested.

Conventional amplification of the 16S rRNA and 56kDa genes was unsuccessful using previously described assays routinely used within our laboratory^{127, 214}. With the use of a different set of 16S rRNA primers, a multiple mixture of various 56kDa primers and custom designed 47kDa primers, we were able to successfully amplify all three genes. The amplified genes showed a level of diversity noticeably greater than any previously identified strain of *O. tsutsugamushi*, which helps to explain the difficulties with initial amplification. For example, it is noted that the majority of *O. tsutsugamushi* strains have less than a one percent divergence with their 16S rRNA sequences, and that *O. tsutsugamushi* strain Shimokoshi was considered the most divergent as it had a percent divergence of around one and a half percent. In contrast this new isolate has a percent divergence with its 16S rRNA gene of two percent and sits well outside the normal *O. tsutsugamushi* cluster (Figure 14).

This leads us to propose that this new isolate be considered a new species of *Orientia*. However, as this genus only currently contains the single species (*O. tsutsugamushi*) there are no established rules to define a new species, unlike that of its close relative the genus *Rickettsia*. Published in 2003, these criteria

specify that a 16S rRNA sequence divergence of greater than 1.2% is one of the conditions for naming a rickettsial isolate as a new species⁸¹. When comparing the percentage divergence of *O. chuto* to nine *O. tsutsugamushi* strains, the percentage divergence of 1.5 – 2.0% falls outside of the 1.2% threshold defined for a novel rickettsial species. However, the second most divergent strain, *O. tsutsugamushi* strain Shimokoshi, has a percentage divergence range of 0.9 – 1.1% (excluding *O. chuto*), which is well within the species threshold mentioned above (Table 9). Therefore, these rules for defining a novel rickettsial species seem to hold true for the *Orientia* genus as all strains fall within the one species with the exception of *O. chuto*. Although no criteria have been established to define a new *Orientia* species, the unique molecular sequences combined with the geographically unique origin of this isolate lead us to claim that it constitutes a new *Orientia* species. Consequently we propose the new species name *Orientia chuto*, with “chuto” being Japanese for “Middle East” with the prototype strain of this species being strain Churchill named after the patient from whom it was isolated.

Sequencing of the 56kDa protein gene of *O. chuto* has proved difficult as it appears to be very divergent from the other strains. However, this will be performed outside of this thesis.

Further studies into this isolate are required to definitively name it as a novel species. These include serological analysis including cross reacting monoclonal

studies, western blotting and possibly genome sequencing. Furthermore, agreed criteria are needed to define a new *Orientia* species.

Chapter 8. *Anaplasma platys* in Australian dogs detected by a novel real-time PCR assay.

This chapter has been submitted for publication to the *Australian Veterinary Journal*. Changes have been made to the formatting of this chapter to integrate it into the thesis.

8.1. Abstract

A specific real-time PCR (qPCR) assay that targeted the 16S rRNA gene of *Anaplasma platys* was designed to test for the prevalence of the agent in the blood of domestic and free roaming dogs in Western Australia, Northern Territory, Queensland and New South Wales. Overall 40% (27/68) of the dogs tested were positive for *A. platys* DNA in their blood, including six dogs from Western Australia. This is the first report of *A. platys* in Western Australia.

8.2. Introduction

Members of the *Anaplasmataceae* family are well known veterinary pathogens, and a number of species including *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* are also known to cause human disease. In Australia there are only three known species of *Anaplasma*; *A. marginale*, *A. centrale*²¹⁵ and *A. platys*³³, the first two cause disease in cattle, while *A. platys* causes disease in dogs.

First identified in 1978 in the United States of America, *A. platys* is an obligate intracellular organism that infects the megakaryocytes of dogs causing canine

infectious cyclic thrombocytopenia¹⁰². It was initially believed that *A. platys* was not present in Australia, however in 2001, Brown *et. al.* detected its presence in free roaming dogs and their ticks in the Northern Territory using molecular methods³³. Worldwide, *A. platys* is transmitted by *Rhipicephalus sanguineus* ticks¹⁰², although in Australia the dog chewing louse (*Heterodoxus spiniger*) has also been shown to harbour this organism³⁴.

Anaplasma platys infected animals typically do not appear unwell, so detection of the disease is often difficult³³. Frequently used staining methods such as Giemsa staining have limited success for detecting the pathogen in blood films. This is due to the cyclic nature of the disease, which often results in low bacterial numbers³². Immunofluorescence assays for antibody detection are also often used. However, as *Anaplasma platys* has never been isolated or cultured, the supply of a standardized antigen is not available and infected dog blood must be used³². There is also a level of cross reactivity and results of serological assays lack specificity⁸⁴. As is the case with other *Rickettsiales*, polymerase chain reaction (PCR) is an effective assay for the detection of *A. platys*¹⁵⁰. This study reports on the use of a novel real time PCR assay to screen the blood of domestic and free roaming dogs in the Northern Territory, Western Australia, Queensland and New South Wales, Australia for the presence of *A. platys*.

8.3. Methods

8.3.1. Probe Design

All available *Anaplasma platys* 16S rRNA gene sequences from the NCBI database (National Center for Biotechnology Information, USA) were aligned using clustalW within the MEGA4 software package²⁴⁵. The consensus sequence obtained was then aligned with the previously published *A. platys* specific primer sequences PLATYS (5' – GAT TTT TGT CGT AGC TTG CTA TG – 3') and EHR16SR (5' – TAG CAC TCA TCG TTT ACA GC – 3') to obtain a 678bp product sequence³³. The qPCR assembly software package AlleleID[®]3 (Premier Biosoft International, USA) was used to design the TaqMan[®] probe EHR16SP (5' – FAM d(CGC CTT CGC CAC TGG TGT TCC TCC) BHQ-1 – 3') that targeted this segment.

8.3.2. Assay Optimisation

The sensitivity and specificity of the new qPCR was determined using methods described in **sections 2.4.2.2 and 2.4.2.1**. Extracted DNA from *Rickettsia honei*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* was used in addition to the controls mentioned in **section 2.4.2.1**.

8.3.3. Sample Preparation

Sixty eight blood samples from dogs were collected in EDTA tubes from ten distinct locations in Australia. Four of the locations were in the Northern

Territory, and two each were within New South Wales, Queensland and Western Australia (Figure 17). All 68 samples were processed using methods described in **sections 2.1.1 and 2.4.1.**

8.3.4. PCR reaction

A DNA sample volume of 2.5 µl was added to a 25 µl reaction mix containing a PCR reaction buffer (Invitrogen, Australia), 0.5 µM of forward and reverse primers, 0.2 µM of probe, 0.625 U Taq DNA polymerase, 0.2 mM deoxyribonucleotide triphosphates and 2 mM MgCl₂. The reaction was performed in a Rotor-Gene 3000 (Corbett Research, Australia), with an initial two minute 95°C incubation, followed by 50 cycles of 94°C for one minute, 54°C for 30 seconds and 72°C for a further 30 seconds.

8.4. Results

8.4.1. Assay Optimisation

The *A. platys* assay was first optimised using a known positive *A. platys* blood sample. The specificity was determined by testing the assay against the other bacterial DNA samples mentioned above. Only DNA from *A. platys* returned a positive result using the assay.

The sensitivity of the assay was determined by using a serial dilution of the *A. platys* 16S rRNA fragment ligated into a TOPO TA plasmid as mentioned

above. The assay was able to detect *A. platys* DNA down to three copy numbers per micro litre (Figure 16).

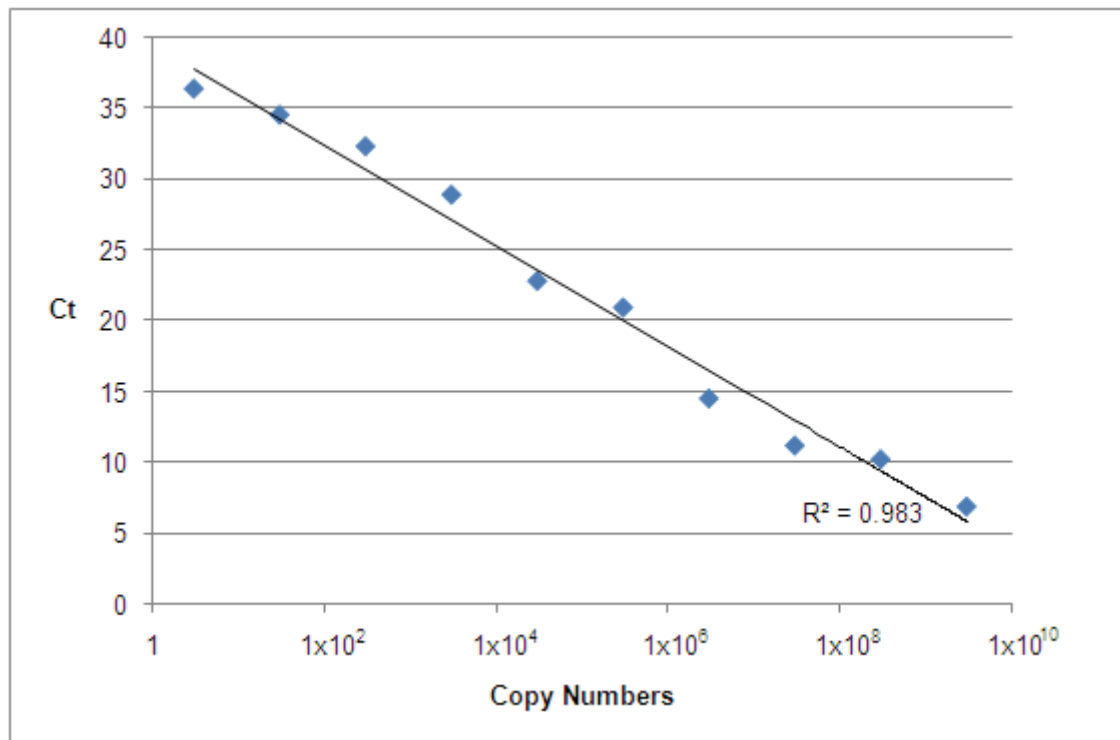


Figure 16. Standard curve showing the relative C_t versus the number of copies of the template containing plasmid.

8.4.2. PCR Results

Of the 68 dogs screened, 27 (40%) were found to be positive for *A. platys* using the qPCR. Of the samples collected from four Northern Territory locations, 13/17 (Ngulu), 6/14 (Yuendumu), 1/2 (Ti Tree) and 0/1 (Darwin) were positive for *A. platys*, 6/13 (Bidyadanga) and 0/1 (Coral Bay) were positive in Western Australia, 1/1 (Monto) and 0/2 (Brisbane) were positive in Queensland. None of

the 17 samples collected in the two New South Wales locations (Goodooga and Collarenebri) were positive for *A. platys* (Figure 17).

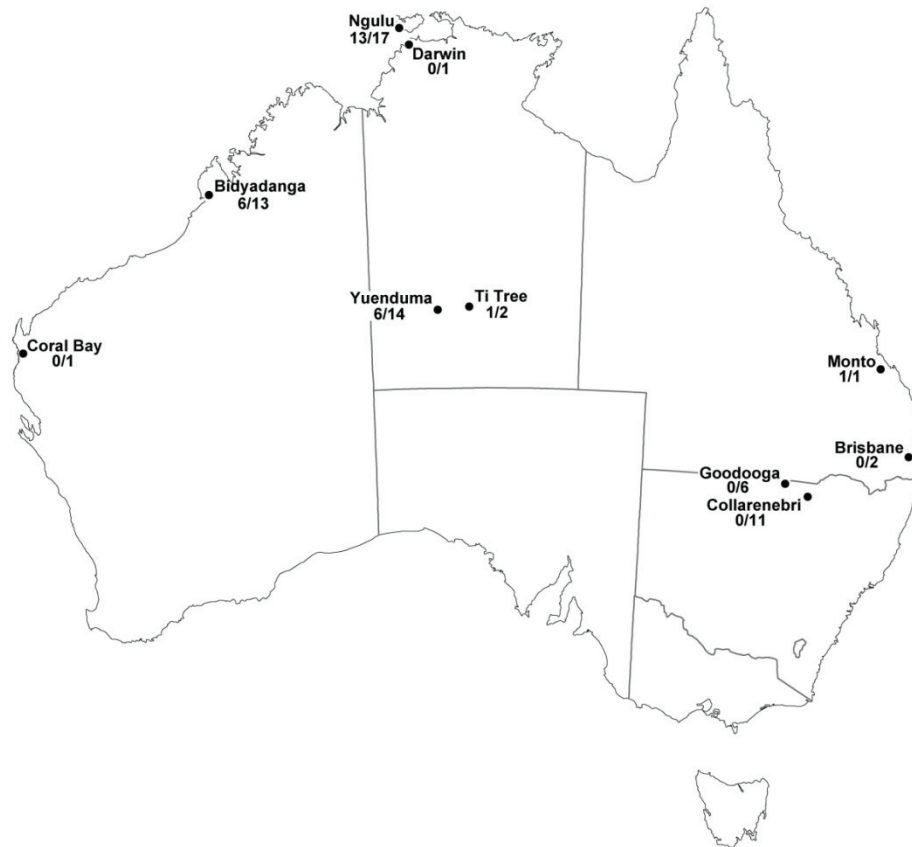


Figure 17: The geographic distribution and number of positive and total *A. platys* samples collected in Australia.

8.5. Discussion

The cyclic nature of *A. platys* infections makes it difficult to detect the bacterium in blood films and cross reactivity of antibodies may lead to false positive diagnosis. The lack of antibodies in recent infections can result in false negative results using IF¹⁰¹. A conventional PCR assay specifically targeting the 16S

rRNA gene of *A. platys* was published in 2001³³, which allowed a much higher level of sensitivity and specificity compared to previously used diagnostic methods³². More recently, real time PCR assays have been used in the detection of rickettsial agents²³⁸ as they typically have increased sensitivity and specificity and are less time consuming than conventional PCR assays. Unlike conventional PCR, real time PCR assays can be semi-quantitative, as an approximate number of copies of the target gene can be determined, and therefore the approximate number of bacteria present can be determined.

This study tested dogs in the Northern Territory, New South Wales, Queensland and Western Australia, with testing sites in ten distinct areas of Australia (Figure 17). While the number of sampling sites and samples that were tested at each site were too small for any patterns to be considered significant, the percentage of infected dogs appeared to increase the further north the dogs resided, with no positive results in northern New South Wales, southern Queensland or the Pilbara region of Western Australia, 44% positive in the central region of Australia, 46% in the Kimberly region of Western Australia and 77% positive in the northern tip of the Northern Territory.

Overall, 27 of 68 (40%) dogs tested were positive for the presence of *A. platys* DNA. These results are comparable to previous studies into the prevalence of *A. platys* in Australia, which showed 13 of 28 (46%)³³ and 93 of 215 (43%)³² of the dogs tested being sero-positive for *A. platys*. This current study failed to detect any *A. platys* in dogs from NSW although, in 2006, Brown *et. al.*³²

detected the agent in the blood of 39% (14/36) of dogs. Although the dogs tested in New South Wales were negative for *A. platys*, 6 of the 13 dogs tested in Western Australia were positive for the presence of *A. platys* in their blood. This is the first time *A. platys* has been reported in Western Australia and indicates that the true range of *A. platys* infection in Australia may be much broader than originally suspected.

This data highlights the need for further research into *A. platys* in Australia. A larger epidemiology study, including southern Australia and areas with higher population densities may help to determine the true range of *A. platys* in Australia.

The majority of the samples including twenty six of the 27 *A. platys* positive dogs were from Aboriginal communities, where both dogs and humans live in very close proximity. This leads to the question as to whether *A. platys* may be a potential zoonotic agent responsible for some of the ill-health in rural Aboriginal people. Studies will need to be undertaken on the blood of Aboriginals in communities with infected dogs to test for the presence of *A. platys* infections.

Testing the blood of other animals and humans within endemic areas for the presence of *A. platys* would help determine whether this organism poses a health threat to the human populations in these regions.

Chapter 9. Concluding Remarks

Little is known of the range and diversity of rickettsial species in Australia. This study began with the screening of antibodies to spotted fever group rickettsial agents in domestic cats and dogs around the city of Launceston in Tasmania.

Of the 368 dog and 150 cats tested, 57% of the dogs and 59% of the cats were positive for the presence of rickettsial antibodies; however there was no statistically significant relationship between positive serology and ill health among either the dogs or cats.

It would be fruitful to expand the sample size and look at different animal species as animal serology may be a good indicator of human infection. Furthermore, additional sampling from humans may allow the extent of rickettsial exposure to be determined in Tasmania.

As there was clear evidence of exposure to an unknown spotted fever group rickettsia in the sero-survey, investigations were undertaken to look for the rickettsial agents. *Ixodes tasmani* ticks were chosen as a possible vector as they are the most abundant tick in Tasmania and are indiscriminate feeders. Forty four *I. tasmani* ticks were collected from Tasmanian devils along the east side of the island. Of the ticks collected, 55% tested positive for the presence of a rickettsial agent using a real time PCR assay. Sequencing of the *gltA*, *rompA*, *rompB* and *sca4* genes identified the agent as a novel SFG rickettsia. Isolation was attempted but was unsuccessful. This novel species was subsequently

named *Candidatus Rickettsia tasmanensis* after the location from which it was detected. To validate this agent as a novel species, further study is required. This includes isolating and growing the agent for submission to a culture collection. Due to the difficulties with isolation of the agent, the *rrs* gene remains to be sequenced. Other isolation techniques could be undertaken including attempting isolation through an animal model for example SCID mouse inoculation.

As with the sero-epidemiological study of domestic animals, this study was limited in its geographical scope, with sampling points only along the east side of the island. Examining *I. tasmani* ticks from the western half of Tasmania, as well as other regions within Australia, would help to determine the true geographical range of this rickettsia.

Testing the blood of animals with *I. tasmani* tick parasitism, using both molecular and serological techniques may provide data on the pathogenic potential of this rickettsia.

Following the initial discovery of this novel rickettsia, other Australian tick species were explored to search for additional novel intracellular or rickettsial species. Ten *Argas dewae* ticks were collected from the roosting boxes of various species of microbats in southern Victoria, of which seven (70%) were positive for rickettsial DNA using a specific real time PCR. Isolation of the agent was successfully achieved using the VERO cell line. Sequencing of the *rrs*, *gltA*, *rompA*, *rompB* and *sca4* genes identified the isolate as a novel SFG rickettsia,

which has been tentatively named *Rickettsia argasii* sp. nov. after the tick species from which it was isolated. All 10 tick samples were collected from roosting boxes in a single geographic location north of Melbourne, Victoria. Bats are known to travel great distances in search of food, so could theoretically spread ticks infected with this novel rickettsial agent over considerable distances. Therefore it would be useful to examine *A. dewae* ticks from roosting boxes in other locations throughout Victoria and other states of Australia for the presence of this agent. This would allow us to determine the true range of this novel species. It would also be useful to test the blood of bats inhabiting the nesting boxes where *R. argasii* positive ticks were found to look for evidence of SFG rickettsial exposure and also to test for the agent itself in bats using molecular and cell culture isolation methods. This would provide data on whether the bats have been exposed to this agent and if so, whether they were actively infected with *R. argasii*. Additionally, humans that have been exposed to these ticks could also be tested for evidence of exposure to *R. argasii* as other *Argas* spp. ticks are known to bite humans. Anecdotal evidence implies that *Argas dewae* is non-pathogenic in SCID mice.

Both *Candidatus R. tasmanensis* and *R. argasii* have currently only been detected in arthropod vectors. However, we don't know if they or other novel rickettsial species in Australia may cause human infection.

A cluster of cases of human rickettsial infection originating in Lara, Victoria were identified. The causative agent, *R. felis*, was previously detected only in flea

vectors within Australia. Serendipitously we recognised five cases of *R. felis* human infection, following exposure to a flea infested kitten from Lara. All five patients had high levels of antibodies to TG rickettsiae, with one patient showing a clear sero-conversion. Furthermore, fleas removed from the group of cats from which the kitten originated, were found to be positive for *R. felis*. This was the first reported human infection with *R. felis* in Australia and the first documented detection of *R. felis* in Victoria. Epidemiological studies of the human populations surrounding the focus point of this study could be undertaken to determine whether this was an isolated cluster of cases or if *R. felis* infections occur in this region but are not diagnosed. Further studies could be expanded to include the whole of Australia. These studies could include putative *R. typhi* (murine typhus) cases, to determine if *R. felis* cases have been incorrectly diagnosed as *R. typhi* due to serological cross reactions, as has been previously reported¹⁸⁴. Cell culture isolation from fleas failed, however, isolation from patient leukocytes could also be attempted in order to isolate an Australian strain of *R. felis*.

As Australia is a large island separated from all other land masses by water, several unique rickettsiae appear to have evolved independently from other rickettsiae worldwide. With the colonisation of the country first by the indigenous people around forty thousand years ago, and more recently by European settlers, together with increased travel and trade, rickettsial agents could have been periodically imported into Australia, as was the case with a patient returning from Dubai in the United Arab Emirates.

A new species of *Orientia* was isolated from the patient's blood. It is unusual to find *Orientia* outside the area of known endemicity. Molecular analysis of the 16S rRNA and 47kDa genes of the isolate showed percentage similarities of 98.5% and 82.3% respectively compared with other strains of *Orientia tsutsugamushi*, which has until now been the only species within the genus. These results indicate that this isolate has evolved quite independently from other known *Orientia tsutsugamushi* strains. However, further characterisation is required in order to establish the phylogenetic position within this family. No criteria have yet been established to define a new *Orientia* species. Protein analysis such as western blotting could also be used to further reinforce the divergence of this organism by comparing it antigenically to other strains of *O. tsutsugamushi*. This new isolate is not only unique in its degree of molecular divergence but also in its geographic origin. Further studies could be undertaken in the region that the patient contracted this disease to try and elucidate the vector and to test the local populace for evidence of exposure to this agent.

While little is known of the diversity of rickettsial species in Australia, even less is known about other members of the *Rickettsiales* including *Ehrlichia* and *Anaplasma*. This study looked for the presence of other members of the *Rickettsiales* within Australia. The presence of *Ehrlichia* in various blood and vector samples were tested, however, as none of the samples contain *Ehrlichia*, no further research was undertaken with this genus. Blood from dogs in aboriginal communities within Western Australia, New South Wales and the

Northern Territory were tested for the presence of *Anaplasma platys* DNA. Of the 68 dogs tested, 27 (40%) were positive for *A. platys* DNA, with increasing rates of positivity the further north the dog populations were located. In our study, none of the 17 dogs tested in New South Wales were positive, unlike an earlier study³². However, 6 of the 14 dogs tested in Western Australia were positive for *A. platys*. This is the first report of *A. platys* in Western Australia. As 26 of the 27 positive results were from Aboriginal communities, where dogs live in very close proximity to humans, this rickettsial agent may have the potential to cause health implications among the communities. Furthermore, testing the blood of the local populace would help determine if this agent is causing health problems. It is important to note however, that with this study, the majority of samples were collected from Aboriginal communities; therefore these results are likely to be biased. Further studies of dogs from other communities would determine whether these levels of positivity were common among other Australian dogs.

Overall, these studies highlight a number of rickettsiae and rickettsial diseases not previously documented in Australia, involving both native and imported animal species as well as humans. It is very likely, many more species remain to be discovered.

Appendices

Appendix 1: Mathematical determination of copy numbers

$$x \text{ ng}/\mu\text{l} = x \text{ }\mu\text{g}/\text{ml}$$

Where x is determined using the ND-1000 spectrophotometer.

- Convert μg to pmol

$$\text{pmol} = x \text{ }\mu\text{g}/\text{ml} \times \frac{10^6 \text{ pg}}{1 \text{ }\mu\text{g}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{N}$$

$$= y \text{ pmol}/\text{ml}$$

Where $660 \text{ pg}/\text{pmol}$ is the average molecular weight of a nucleotide pair and N is the size in base pairs of the vector/insert complex.

- Convert pmol/ml to copies/ml

$$\text{copies}/\text{ml} = \left(y \text{ pmol}/\text{ml} \times \frac{10^{-12} \text{ mol}}{1 \text{ pmol}} \right) \times 6.022 \times 10^{23} \text{ copies}/\text{mol}$$

$$= z \text{ copies}/\text{ml}$$

To determine the number of copies, Avogadro's number needs to be used, where one mole of something consists of 6.022×10^{23} units of that substance.

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